METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF TELMISARTAN AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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CERTIFICATE

This is to certify that the dissertation work entitled "**METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF TELMISARTAN AND HYDROCHLOROTHIAZIDE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC**" is a bonafide work of **Ms. KOUMUDI ANNAMANENI** carried out in AUROBINDO PHARMA LABORATORIES, HYDERABAD under my guidance and under the supervision of Ms. P.USHA RANI Senior research Analyst and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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INTERNAL EXAMINER

Place:

Date:

EXTERNAL EXAMINER

Place:

Date:

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It gives me immense pleasure to convey my deep sense of gratitude and heartfelt thanks to my guide **Mr. P.KUMAR NALLASIVAN,** Assistant Professor, Department of Pharmaceutical Analysis, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore for his help, suggestions, guidance, and the confidence that he has shown in me throughout the course of my work.

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KOUMUDI ANNAMANENI

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ABBREVIATIONS

ACN	:	Acetonitrile
g	:	gram
HPLC	:	High performance liquid chromatography
pH	:	Hydrogen ion concentration
ICH	:	International conference on harmonization
mg	:	Milligram
ml	:	Milliliter
µg	:	Microgram
µg/ml	:	Microgram per milliliter
µg	:	Microgram
nm	:	Nanometer
PA	:	Purity Angle
PDA	:	Photo diode array
TH	:	Purity threshold
RSD	:	Relative standard deviation
k	:	Retention time
SD	:	Standard deviation
UV	:	Ultra violet
V/v	:	Volume by Volume
%DEG	:	% Degradation

CONTENTS

1. INTRODUCTION

1.1 CHROMATOGRAPHY[1]:

Chromatography is a valuable technique for the separation, purification, and identification of the constituents in a mixture. This technique was originally confined to the seperation of coloured substances such as plant pigments and dyestuffs. But the technique is now well applied to colourless substances also.

The chromatographic technique was first invented by M.TSWETT in 1906. The term chromatography (greek :- khromatos – colour and graphos – written) and its principles were first discovered by Michel Tswett .

On the general progress of science, chromatography may be regarded as an analytical technique employed for the purification and separation of organic and inorganic substances. It is also found useful for the fractionation of complex mixture, separation of closely related compound such as isomers and in the isolation of unstable substances.

Principles of chromatographic separation[2]:

- Adsorption chromatography: A solid stationary phase and a liquid or gaseous mobile phase.
- Partition chromatography: A liquid stationary phase and a liquid or gaseous mobile phase.
- Ion exchange chromatography: A solid polymeric stationary phase containing replaceable ions.
- Size exclusion chromatography: An inert gel which acts as a molecular sieve, and liquid mobile phase.

Adsorption chromatography:

In adsorption chromatography, the mobile phase containing the dissolved solutes which are passed over the surface of stationary phase, due to the affinity of the solute towards mobile or stationary phase, solutes gets separated. In normal phase the mobile

phase is non polar and the stationary phase is polar where in reverse phase the mobile phase is polar and the stationary phase is non polar.

Partition chromatography:

In partition chromatography the principle of separation is readily understood by considering the partitioning behaviour of substance between two immiscible liquids. Few substances, when shaken with two immiscible liquids, partition take place completely in to one or other liquid. Instead, most distribute them selves between the liquids such that the partition coefficient (the ratio of concentrations of the substance in each phase) is a constant value independent of the total amount, provided neither phase is saturated with the substance.

Mode of chromatographic operations[3]:

There are three modes of chromatographic operation they are as follows:

- Elution techniques
  - Isocratic method
  - Gradient method
- Frontal techniques
- Displacement techniques

Types of chromatography techniques:

- Planar chromatography
- Column chromatography

TYPES OF LIQUID CHROMATOGRAPHY[4]

- Liquid – Solid chromatography
- Liquid – liquid chromatography
- Gas liquid chromatography

These three types are the basic types of chromatography and these are modified to different types of chromatography. They are as follows:

- Normal phase chromatography
- Reverse phase chromatography
- High performance liquid chromatography
- Ion exchange chromatography
- Size exclusion liquid chromatography
- Super critical fluid chromatography
- Chiral chromatography
- Affinity chromatography

1.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[5]:

The modern form of column chromatography has been called high performance, high pressure, and high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

HPLC has the following important features.

- High resolution power
- Speedy separation
- Continuous monitoring of the column effluents
- Accurate quantitative measurement
- Repetitive and reproducible analysis, using the same columns

Automation of the analytical procedure and date handling

Normal Phase Chromatography

In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents.

The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.

- Dipole-induced dipole
- Dipole-dipole
- Hydrogen bonding
- π-Complex bonding

These situations arise when the molecule has one or several atoms with lone pair electron or a double bond. The absorption strengths and hence k' values (elution series) increase in the following order. Saturated hydrocarbon < olefins < aromatics < organic halogen compounds < sulphides < ethers< esters < aldehydes and ketones < amines < sulphones < amides < carboxylic acids. The strength of interactions depends not only on the functional groups in the sample molecule but also on steric factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.

Chemically modified silica, such as the aminopropyl, cyanopropyl and diol phases is useful alternatives to silica gel as stationary phase in normal phase chromatography.

The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the stationary phases and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in their increased homogeneity of the phase surface.

Resolution with water in weak mobile phase may be most conveniently achieved by drying the solvents and then adding a constant concentration of water or some very polar modifier such as acetic acid or trietylamine (TEA) to the mobile phase. The addition of such polar modifiers serves to deactivate the more polar shape as well as the reproducibility of the retention times.

Reversed Phase Chromatography

In 1960's chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

A large number of chemically bonded stationary phases based on silica are available commercially. Table 1 lists some of the functional groups bonded in chemically modified silica. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-divinyl benzene copolymer) are slowly gaining ground.

Simple compounds are better retained by the reversed phase surface, the less water-soluble (i.e. the more non-polar) they are. The retention decreases in the following order: aliphatics > induced dipoles (i.e.,CCl_4) > permanent dipoles (e.g.CHC_{l3}) > weak lewis bases (ethers, aldehydes, ketones) > strong lewis bases (amines) > weak lewis acids (alcohols, phenols) > strong lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e., with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reversed phase systems the strong attractive forces between water molecules arising from the 3-dimentional inter molecular hydrogen bonded network, from a structure of water that must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Non- polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with the hydrocarbon moieties of the stationary phase.

Chemically bonded octadecyl silane (ODS) an alkaline with 18 carbon atoms it is the most popular stationary phase used in pharmaceutical industry. Since most

pharmaceutical compounds are polar and water soluble, the majority of HPLC methods used for quality assurance, decomposition studies, quantitative analysis of both bulk drugs and their formulations use ODS HPLC columns. The solvent strength in reversed phase chromatography is reversed from that of adsorption chromatography (silica gel) as stated earlier. Water interacts strongly with silanol groups, so that adsorption of sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reversed phase system; water cannot wet the non-polar (hydrophobic) alkyl groups such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reversed phase chromatography increases with increasing amount of water in the mobile phase.

The essential equipment consists of an eluent, reservoir, a high-pressure pump, and an injector for introducing the sample, a column containing the stationary phase, a detector and recorder. The development of highly efficient micro particulate bonded phases has increased the versatility of the technique and has greatly improved the analysis of multicomponent mixtures.

The systems used are often described as belonging to one of four mechanistic types, adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase. Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first.

The various components of a HPLC system are herewith described.

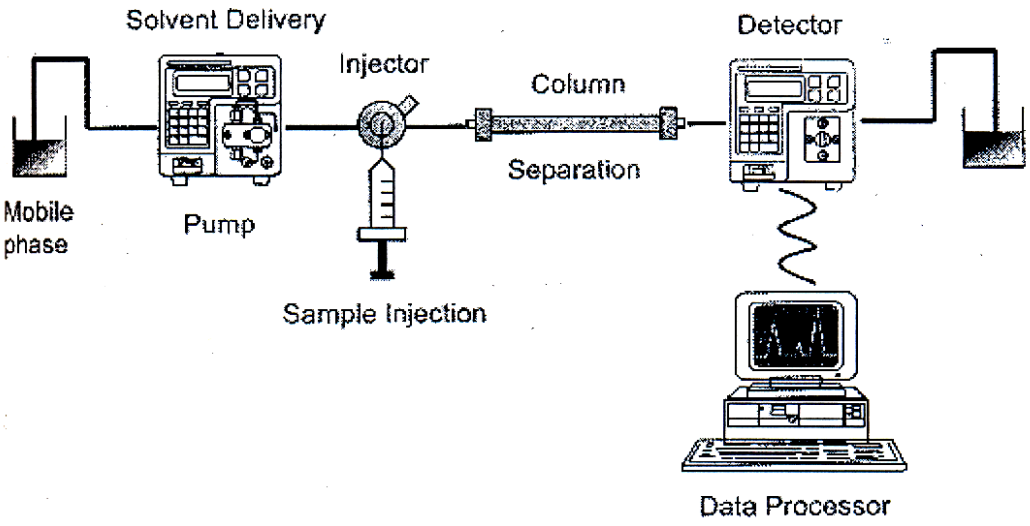


Fig.No.1. Schematic diagram of the HPLC system

Instrumentation[6,7]

Solvent delivery system

The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of the solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

The most important component of HPLC in solvent delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.) reciprocating pump with twin or triple pistons is widely used, as this system gives less baseline noise, good flow rate reproducibility etc.

Solvent degassing system

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45µ filter, vacuum degassing with an air-soluble membrane, helium purging ultra sonication or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

Gradient elution devices

HPLC columns may be run isocratically, i.e., with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during run. Gradient elution is a means of over coming the problem of dealing with a complex mixture of solutes.

Sample introduction systems

Two means for analyte introduction on the column are injection in to a flowing stream and a stop flow injection. These techniques can be used with a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of the manual universal injector. Usually, up to 100 samples can be loaded in to the auto injector tray. The system parameters such as flow rates, gradient, run time, volume to be injected, etc. are chosen, stored in memory and sequentially executed on consecutive injections.

Liquid chromatographic detectors[8,9]

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Generally, there are two types of HPLC detectors, bulk property detectors and solute property detectors.

Bulk property detectors

These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors.

Solute property detectors

Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase. These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV or UV-Vic) detector, fluorescence detectors, polarographic, electro-chemical and radio activity detectors, whilst the moving wire flame ionization detector and electron capture detector both require removal of the mobile phase before detection.

UV-Vis and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent.

Column and Column-packing materials[10]

The heart of the system is the column. In order to achieve high efficiency of separation, the column material (micro-particles, 5-10 μm size) packed in such a way that highest numbers of theoretical plates are possible.

Silica (SiO_2,H_2O) is the most widely used substance for the manufacture of packing materials. It consists of a network of siloxane linkages (Si-O-Si) in a rigid three dimensional structure containing inter connecting pores. Thus a wide range of commercial products is available with surface areas ranging from 100 to 800 m^2/g. and particle sizes from 3 to 50 μm.

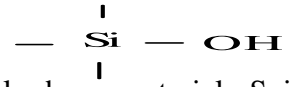
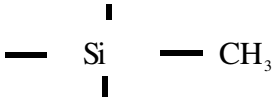
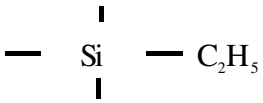
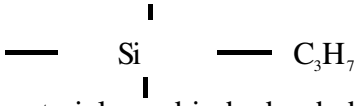
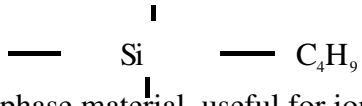
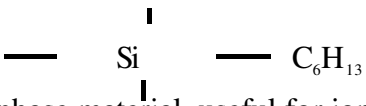
The silanol groups on the surface of silica give it a polar character, which is exploited in adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organo chloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with the surface. The attachment of hydrocarbon change to silica

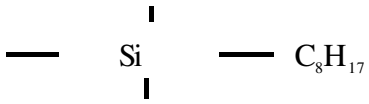
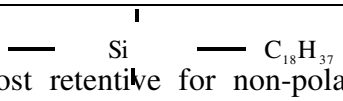
produces a non-polar surface suitable for reversed phase chromatography where mixtures of water and organic solvents are used as eluents. The most popular material is octadecyl-silica (ODS-Silica), which contains C_{18} chains, but materials with C_2, C_6, C_8 and C_{22} chains are also available. During manufacture, such materials may be reacted with a small mono functional silane (e.g. trimethyl chloro silane) to reduce further the number of silanol groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding to silica of other organic compounds which contain groups such as phenyl, nitro, amino and hydroxyl. Strong ion exchangers are also available in which sulphonic acid groups or quaternary ammonium groups are bonded to silica. The useful pH range for columns is 2 to 8, since siloxane linkages are cleaved below pH-2 while at pH values above eight silica may dissolve.

In HPLC, generally two types of columns are used, normal phase columns and reversed phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on a stationary silica phase. However, it now seems that partition plays an important role, with the compounds interacting with the polar silanol groups on the silica or with bound water molecules.

While normal phase seems the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar mobile phase such as methanol, Acetonitrile, water, buffers etc., over a non-polar stationary phase. Ranges of stationary phases (C_{18}, C_8, -NH_2, -CN, -phenyl etc.) are available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of the drug and thereby increase the retention on the column. For highly ionized drugs ion-pair chromatography is used.

Table 1.Bonded Phases for HPLC and their Abbreviations

Phase	Description
Si	Silica  Classic normal phase material. Suitable for separating polar non-ionic organic compounds.
C_1	TMS, SAS, Trimethyl silane  Reversed phase material. Unique selectivity for polar and multifunctional compounds. Least retentive of all alkyl group bonded phases for non-polar solvents.
C_2	RP-2, Dimethyl  Reversed phase material, less retentive than C_4, C_8, or C_{18}. More retentive than C_1.
C_3	Propyl  Reversed phase material, used in hydrophobic interaction chromatography (HIC) of proteins and peptides.
C_4	Butyl  Reversed phase material, useful for ion-pairing chromatography offers less retention than C_8 and C_{18} phases for non-polar solutes. When bonded to 300 Å silica, it is an ideal phase for analyzing large proteins and hydrophobic peptides.
C_6	Hexyl  Reversed phase material, useful for ion-pairing chromatography. Less retentive than C8 and C18 phases.

C_8	MOS, RP-8, LC8, Octyl  Reversed phase material, similar selectivity to C18 but less retentive. Wide applicability (e.g. pharmaceuticals, nucleosides, steroids). When bonded to300 Å silica, it is an ideal phase for peptides, peptide mapping and small hydrophilic proteins.
C_{18}	ODS, RP-$_{18}$, LC$_{18}$, Octadecyl  Classic reversed phase material is most retentive for non-polar solutes and is excellent for ion-pairing chromatography. It is having wide applicability for the assay of nucleosides, nucleotides, steroids, pharmaceuticals, vitamins, fatty acids and environmental compounds when bonded to 300 Å silica, this phase is perfect for separating small hydrophilic peptides.

Derivatization

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chlorides, while fluorescent derivatives can be formed with reagents such as dansyl chloride, 4-bromo methyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected on to the column or by online chemical reactions between the column out let and the detector.

Gradient elution

Gradient elution or solvent programming is the change of solvent composition during a separation in which the solvent strength increases from the beginning to the end of the separation. It is well suited to the analysis of samples of unknown complexity since good resolution is automatically provided for a wide range of

sample polarities. There are two types of gradient systems: Low-pressure gradient mixtures and high- pressure gradient mixtures. In the former the solvents are mixed at atmosphere pressure and then pumped to the column, where as in the later, solvents are pumped in to a mixing chamber at high pressure before going in to the column.

1.3. METHOD DEVELOPMENT AND OPTIMIZATION[11]

During the optimization stage, the initial sets of conditions that have evolved from the first stages of development are improved or maximized in terms of resolution and peak shape, plate counts asymmetry, capacity, elution time, detection limits, limit of Quantitation, and overall ability to quantify the specific analyte of interest.

Optimization of a method can follow either of two general approaches:

1. Manual
2. Computer driven

The manual approach involves varying one experimental variable at a time, while holding all others constant, and recording changes in response .The variables might include flow rates, mobile or stationary phase composition, temperature, detection wavelength, and pH this univariate approach to system optimization is slow, time consuming and potentially expensive. However, it may provide a much better understanding of the principles and theory involved and of interactions of the variables.

In the second approach, computer driven automated methods development, efficiency is optimized while experimental input is minimized. Computer driven automated approaches can be applied to many applications .In addition, they are capable of significantly reducing the time, energy and cost of virtually all-instrumental methods development.

The various parameter s that include to be optimized during method development [12]

1. Mode of separation
2. Selection of stationary phase
3. Selection of mobile phase
4. Selection of detector

1.3.1 Selection of mode of separation

In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix.

1.3.2. Selection of stationary phase / column

Selection of the column is the first and the most important step in method development the appropriate choice of separation column includes three different approaches

1. Selection of separation system
2. The particle size and the nature of the column packing
3. The physical parameters of the column i.e. the length and the diameter

Some of the important parameters considered while selecting chromatographic columns are

- Length and diameter of the column.
- Packing material.
- Shape of the particles.
- Size of the particles.
- % of Carbon loading
- Pore volume.
- Surface area.
- End capping.

The column is selected depending on the nature of the solute and the information about the analyte. Reversed phase mode of chromatography facilitates a wide range of columns like dimethyl silane (C_2), butylsilane (C_4), octylsilane (C_8), octadecylsilane (C_{18}), base deactivated silane (C_{18}) BDS phenyl, cyanopropyl (CN), nitro, amino etc. C_{18} was chosen for this study since it is most retentive one. The sample manipulation becomes easier with this type of column

Generally longer columns provide better separation due to higher theoretical plate numbers. As the particle size decreases the surface area available for coating increases. Columns with 5-µm particle size give the best compromise of efficiency, reproducibility and reliability. In this case, the column selected had a particle size of 5 µm and a internal diameter of 4.6 mm

Peak shape is equally important in method development. Columns that provide symmetrical peaks are always preferred while peaks with poor asymmetry can result in,

- In accurate plate number and resolution measurement
- Imprecise quantitation
- Degraded and undetected minor bands in the peak tail
- Poor retention reproducibility

A useful and practical measurement of peak shape is peak asymmetry factor and peak tailing factor. Peak asymmetry is measured at 10% of full peak height and peak tailing factor at 5%. Reproducibility of retention times and capacity factor is important for developing a rugged and repeatable method.

A column which gives separation of all the impurities and degradants from each other and from Analyte peak and which is rugged for variation in mobile phase shall be selected.

1.3.3. Selection of mobile phase

The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from each other and from analyte peak

In liquid chromatography, the solute retention is governed by the solute distribution factor, which reflects the different interactions of the solute – stationary phase, solute – mobile phase and the mobile phase – stationary phase .For a given stationary phase, the retention of the given solute depends directly upon the mobile

phase, the nature and the composition of which has to be judiciously selected in order to get appropriate and required solute retention. The mobile has to be adapted in terms of elution strength (solute retention) and solvent selectivity (solute separation) Solvent polarity is the key word in chromatographic separations since a polar mobile phase will give rise to low solute retention in normal phase and high solute retention in reverse phase LC. The selectivity will be particularly altered if the buffer pH is close to the pKa of the analytes; the solvent strength is a measure of its ability to pull analyte from the column. It is generally controlled by the concentration of the solvent with the highest strength.

The following are the parameters, which shall be taken into consideration while selecting and optimizing the mobile phase.

- Buffer
- pH of the buffer
- Mobile phase composition.

1.3.3.1. Buffer, if any and its length.

Buffer and its strength play an important role in deciding the peak symmetries and separations. Some of the most, commonly employed buffers are

- Phosphate buffers prepared using salts like KH_2PO_4, K_2HPO_4,NaH_2PO_4,Na_2HPO_4,etc
- Phosphoric acid buffers prepared using H_3PO_4.
- Acetate buffers – Ammonium acetate, Sodium acetate, etc.
- Acetic acid buffers prepared using CH_3COOH.

The retention times also depend on the molar strengths of the buffer – Molar strength is increasingly proportional to retention times. The strength of the buffer can be increased, if necessary, to achieve the required separations.

The solvent strength is a measure of its ability to pull analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength.

1.3.3.2. pH of the buffer

pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Experiments were con ducted using buffers having different pH to obtain the required separations**.**

It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkage area cleaved below pH 2.0, while pH valued above 8.0 silica may dissolve.

1.3.3.Mobile phase composition:

Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to that fact that fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions. Most widely used solvents in reverse phase chromatography are Methanol and Acetonitrile. Experiments were conducted with mobile phases having buffers with different pH and different organic phases to check for the best separations between the impurities. A mobile phase which gives separation of all the impurities and degradants from each other and from Analyte peak and which is rugged for variation of both aqueous and organic phase by at least ±0.2% of the selected mobile phase composition.

1.3.4. Selection of detector:

The detector was chosen depending upon some characteristic property of the analyte like UV absorbance, fluorescence, conductance, oxidation, reduction etc. characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity, facilitating trace analysis
- Negligible baseline noise. To facilitate lower detection
- Large linear dynamic range
- Low dead volume
- Non destructive to sample

- Inexpensive to purchase and operate

Pharmaceutical ingredients do not all absorb UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful.

For the greatest sensitivity λ_{max} should be used. UV wavelengths below 200 nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

VALIDATION OF ANALYTICAL METHOD DEVELOPMENT

Introduction:

Analytical method validation[13,14] is the process of demonstrating that analytical procedures are suitable for their intended use and provide accurate test results that evaluate a product against its defined specification and quality attributes.

It is process involving confirmation or establishing by laboratory studies the method / procedure/ system/ analyte gives accurate and reproducible result for intended application in a proven and established range. That performance characteristic of the method (accuracy, precession, sensitivity, ruggedness etc)..

Types of validation:[15]

Prospective Validation: At least three successive production size (US Via) batches, all batches made tested and report approved before distribution facilities and equipment qualified.

Concurrent Validation: Generation of validation data concurrent or simultaneously with normal production schedules used in exceptional cases (low volume products); interim reports required.

Retrospective Validation: This is establishing documented evidence that the process is performed satisfactorily and consistently over time, based on review and analysis of historical data. The source of such data is production and QA/QC records. The issues to

be addressed here are charged to equipment, process, specification and other relevant changes in the past.

1.4.1 VALIDATION OF ANALYTICAL PROCEDURES [16,17]

Definitions, Methodology and Acceptance Criteria:

Different Types of Validation characteristics:

- Precision
- Accuracy
- Specificity and Selectivity
- Linearity and Range
- Solution stability
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- Ruggedness.
- System Suitability

Generalized validation process for an HPLC assay method:

Validation[2] is the process of collecting documented evidence that the method performs according to its intended purpose. The validation process has been described as follows:

1. Accuracy

Accuracy[18] is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. There are several methods that can be used for determining accuracy. The most common include

Analyze a sample of known concentration and compare the measurement to the true value. In this case, method accuracy is the agreement between the difference in the measured analyte concentration and the known amount of analyte added. That is the accuracy or % recovered is calculated as:

$$\frac{C_m \times 100}{C_t}$$

Where C_m is the measured concentration

C_t is the theoretical concentration.

Accuracy has also been reported as a sample is analyzed and the measured value should ideally be identical to the true value. Accuracy is represented and determined by recovery experiments. The usual range is being 10% above or [below] the expected range of claim. The % recovery was calculated using the formula,

$$\text{Percentage Recovery} = \frac{(a + b) - a}{b \times 100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

ICH states that accuracy should be assessed using a maximum 9 determination over a minimum of 3 concentration levels covering the specified range (eg. 3 concentration /3 replicates each of the total analytical procedure).

Acceptance Criteria:

1. For an assay method, mean recovery will be 100%± 2% at each concentration over the range of 80-120% of the target concentration.

2. For an impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities in the range of 0.1-2.5 % (V/W).

2. Precision:

Precision[14,19] of an analytical procedure expressed the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple samplings of the same homogeneous sample under prescribed condition.

The precision of test method is usually expressed as the standard deviation or relative standard deviation of a series of measurements. Precision may be considered at three levels: *Repeatability*, *Intermediate Precision* and *Reproducibility*.

System precision:

A System precision was evaluated by measuring the peak response of drug for six replicate injection of the standard solution preparation as per the proposed method.

Method precision:

The method precision was determined by preparing the sample of a single batch of the drug for tablet formulation six times and analysed as per the propsed method.

Acceptance Criteria:

1.Percentage Relative standard deviation (%RSD) NMT 1 % (Instrument precision)

2.(%RSD) NMT -2% (Intra- assay precision)

3. Specificity: Specificity[19] is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Specificity shall be demonstrated by performing Placebo / blank interference and forced degradation studies.

a. **Blank interference:**

Blank solution is prepared and analysed as per test method.

b. **Placebo interference (In case of Drug products):**

The placebo solution equivalent to the test concentration is also prepared and analysed as per the test method.

4. Forced Degradation studies:

The sample is degraded forcefully under the various stress conditions like Light, heat, humidity, acid / base / water hydrolysis and oxidation to ensure the degradation ranging 1 % to 20 %.

a) ***Light:*** The Drug product, drug substance and placebo are exposed to UV light for about 200 watt hours / square meter and the overall illumination not less than 1.2 million Lax hours for visible light. The sample and placebo solution are prepared as per test method and analyzed.

b) ***Humidity:*** The Drug product, drug substance and placebo are exposed for about 80% RH at about 25°C for about one week. Prepare the sample and placebo solution as per test method and analyzed.

c) ***Heat:*** The Drug product, drug substance and placebo are exposed at 105°C for about 12 hours (For substance having low melting point below 10°C of its melting point). The sample and placebo solution are prepared as per test method and analyzed.

d) ***Acid / Base:*** The 0.1N acid or base solution of the drug refluxes the sample and placebo with 50 ml of acid / base solution for about 1 hour at 60°C. Neutralize the solution and dissolve the contents in diluents as per test method. Change the strength of acid and base or reflux time to ensure the desired degradation.

e) ***Oxidation:*** The sample was refluxed for 12 hour at 60°C with 1 % H_2O_2 or suitable oxidant and dissolved the contents in diluents as per test method. The reflux time was changed so as to ensure the desired degradation.

f) ***Water:*** The sample / placebo was refluxed with 100 ml of purified water for 12 hour at 60°C. Dissolve the contents in diluents as per test method. Change the reflux time so as to ensure the desired degradation.

Note: Based on the physic-chemical properties and literature stress conditions can be decided.

Acceptance Criteria:

1.Placebo / Blank should not elute at the retention time of analyte peak and known impurity peak.

2.Peak purity of analyte peak should be confirmed.

3.Degradation of active analyte peak should be from 1% to 20%.[23]

5. Limit of Detection:

The limit of detection[20] is the lowest concentration of analyte in a sample that can be detected but not necessarily determined in quantitatively using a specific method under the required experimental conditions. Such a limit is expressed in terms of concentration of analyte in the sample.

Following are different approaches:

1. **Visual Evaluation Method:**

The sample solutions have to be prepared with known lowest concentrations of analyte and establish the minimum concentration at which the analyte can be reliably detected by analyzing as per test method.

a. Based on Signal to Noise Ratio Method:

The LOD can be expressed as a concentration at specified signal-to-noise (S/N) ratio obtained from samples spiked with analyte. A signal-to-noise(S/N) ratio between 3:1 and 2:1 is generally considered acceptable.

b. Based on the standard Deviation of the Response and the Slope:

1. The blank solution has to be prepared as per test method and inject six times into the chromatographic system.**2.** Similarly the linearity solution staring from lowest possible concentration of analyte to 150 % (or as per protocol) of target concentration have to be prepared to establish the linearity curve.

The detection limit (DL) may be expressed as :

$$LOD = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

S= slope of the calibration curve of the analyte.

The slope shall be estimated from the calibration curve of the analyte.

6. Limit Of Quantitation:

The limit of quantitation[20] is the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions quantitation limit is expressed as the concentration of analyte (eg. Percentage, parts per million) in the sample.

Following are different approaches:

a. Visual Evaluation Method:

The sample solutions with known lowest possible concentrations of analyte and establish the minimum concentration at which the analyte can be reliably quantified by analyzing as per test method.

b. Based on signal to noise ratio method :

The LOQ can be expressed as a concentration at specified signal-to-noise ratio obtained from samples spiked with analyte. A signal-to-noise ratio of 10:1 is generally considered acceptable. The ratio recognized by the ICH (1996) is a general rule. It has been stated that "the determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases.

c. Based on the standard Deviation of the Response and the Slope:

The blank solution as per test method and inject six times into the chromatographic system. Similarly the linearity solution staring from lowest possible concentration of analyte to 150% (or as per protocol) of target concentration and establish the linearity curve.

The limit of quantitation (LOQ) may be expressed as :

$$LOQ = \frac{10 \times \text{Standard deviation of the response of the blank}(\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

Acceptance Criteria:

1. In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.

2.LOQ is defined as the lowest concentration providing a RSD of 5%.

3.LOQ should be at least 10% of the minimum effective concentration for clinical applications.

4.Signal -to- noise ratio is 10:1.

7. Linearity and range*:*

Linearity: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

Range: Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrated that the analytical procedure has a sutable level of precision, accuracy, and linearity.

Measurement: A range of standards should be prepared containing at least 5 different concentration of analyte which are approximately evenly spaced and span 50-150% of the label claim.

At least 6 replicates per concentration to be studied. Plot a graph of concentration (on X-axis) Vs mean response (on Y-axis) calculates the regression equation.

Y – Intercept and correlation coefficient. Plot another graph of concentration (on X-axis) Vs response ratio (replicate response divided by concentration on Y-axis).

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy, and linearity when applied to sample containing analyte at the extreme of the range as well as within the range.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

8. Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same sample under a verity of conditions, such as different laboratories, different analysts, different instruments etc.

Normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method.

Ruggedness is a measure of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst

The following are the typical method parameters need to tested during method validation:

- Analyst-to-Analyst variability.
- Column-to-Column variability.
- System-to-System variability.
- Different days.
- Different Laboratories.
- Stability of Solutions and mobile phase. (At least for 48 hours)

9. Robustness:

Robustness of an analytical method is a measure of its capacity ti remain un affectedly small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

For example a chromatographic method, the typical method parameters need to change deliberately and verify during method validation:

Flow rate	:	(+/- 0.2ml/minutes).
Mobile phase composition	:	(+/- 10% of organic phase).
Column oven temperature	:	(+/- 5°C).

P_H of buffer in mobile phase	:	(+/- 0.2 units).
Filter suitability	:	(At least two filters).

For Variations:

1. System suitability should meet the acceptance criteria as per test method.

2. If system suitability doesn't meet, the variation range is narrowed and carried out the experiment again to meet system suitability.

10. SYSTEM SUITABILITY TESTING

System suitability testing[21] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics analytical operation and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

System Suitability Parameters

- Tailing factor
- Theoretical plate number
- Theoretical plate per meter
- Resolution factor
- Capacity factor
- Precision / Injection repeatability
- Relative retention

a. Tailing factor:

Asymmetry factor[13] of a peak was calculated from the following expression:

$$\text{Peak tailing factor} = \frac{A + B}{2A}$$

A – Left half of the peak at 5% peak height when the peak is bisected with a perpendicular line dropped from the maximum of peak interest.

B – Right half of the peak at 5% peak height when the peak is bisected with a perpendicular line dropped from the maximum of peak interest.

b. Theoretical plate number (N):[14]

The assessment of performance of column efficiency of a column is in terms of number of theoretical plate

An equation shown below:

$$N = 5.54[t/wh/2]$$

Where,

t = Retention time

Wh/2 = width of peak at half weight

Theoretical plate number is a measure of column efficiency. i.e. how many peaks can be located per unit run time of the chromatogram ,'H' or 'HETP', the height equivalent to the theoretical plate, measures the column efficiency per unit length of the column. Parameters which can affect 'N' or 'H' include peak position particle size in column, flow rate of mobile phase and molecular weight of the analyte.

c. Capacity factor:[21]

It reflects the location of peak of interest with respect to the void volume i.e. elution time of the unretained compound.

$$K_1 = (t_r - t_o) - t_o$$

[t_r – retention of analyte, t_o – retention of void or unretained component.]

Methods used for the examination of pharmaceutical material may be broadly classified as;

d. Resolution: [17]

$$R = 2\,[(t_2 - t_1)/\, w_2 + w_1]$$

t_2, t_1 are the retention time

w_2, w1 are the peak width at base line respectively.

Resolution is to measure how well the resulting two peaks are separated. For reliable quantitation well separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak may be of concern. The closest eluting peak to the analyte should be selected.

System Suitability Parameters and Recommendations (ICH Guidelines)

Parameter	**Recommendation**

Capacity Factor (k')	the peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Relative retention	not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc).
Tailing Factor (T)	T of </= 2
Theoretical Plates (N)	In general should be > 2000

Class A: Test designed to establish identity, whether of bulk drug substances or particular ingredient in a finished dosage form.

Class B: Method designed to detect and quantitative impurities in a bulk drug substance or finished dosage form.

Class C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form.

Class D: Methods used to assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

Table No: 2 Characteristics that should be considered for different Type of analytical procedure:-

S.No	Parameters	Class A	Class B		Class C	Class D
			Quantitative test	Limit test		

1.	Accuracy	-	Yes	-	Yes	Yes
2.	Precision	-	Yes	-	Yes	Yes
3.	Robustness	Yes	Yes	Yes	Yes	Yes
4.	Linearity and Range	-	Yes	-	Yes	Yes
5.	Selectivity	Yes	Yes	Yes	Yes	Yes
6.	LOD	Yes	Yes	Yes	-	-
7.	LOQ	-	Yes	-	-	-

2. LITERATURE REVIEW & DRUG PROFILE

2.1.LITERATURE REVIEW:

1.V.Amudhavalli et al[22] .,(2001) have done determination of Olmesartan and hydrochlorothiazide in the pharmaceutical formulation by RP-HPLC.A simple and sensitive method was developed on the shimadzu class HPLC system using c_{18} column. Using mobile phase mixture Acetontrile and ammonium acetate buffer(ph-3.5) in the ratio of 55:45.Flow rate was 1.0ml/min and the effluents were monitored at 252nm and eluted at 2.14 and 4.62.The assay was validated for parameters like accuracy, precision, robustness, and system suitability.

2.Sudhakar Nandipati et al[23] .,(2012) have done Development and validation of RP-HPLC method for the estimation of Telmisartan in bulk and tablet dosage form .A simple RP-HPLC method in the bulk dosage form for estimation of Telmisartan has developed .Mobile phase was potassium di-hydrogen phosphate buffer and acetonitrile(60:40) pH adjusted with phosphoric acid,c_{18} sun fire column(250×4.6×5µm) flow rate 1ml/min,wave length 243 nm ,column temperature 45˚c,injection volume 10µl.System suitability parameters of Telmisartan retention time 3.4,plate count 8968,tailing 1.086,%RSD 0.1 those all are within the limit method is suitable for analysis. Validation parameters selectivity, precision, linearity, accuracy ,robustness all are within the limit so method was validated it is use full to the pharmaceutical analysis.

3.Paul Richards M et al[24] .,(2011) have done Simultaneous estimation of Telmisartan and Amlodipine Besylate in the pharmaceutical dosage form. The chromatographic analysis was performed on ODS symmetry c_{18} column(150x4.6mm,5µ particle size) with mobile phase consisting if acetonitrile and phosphate buffer(ph-4.0) in the ratio of 60:40 v/v,at the flow rate of 1.2ml/min and eluents monitored at the 237 nm. The method was validated for linearity ,accuracy ,precision ,robustness and application for assay as per the ICH guidelines.The retention times of amlodipine besylate and telmisartan were 2.633 and 5.6 min, respectively.The proposed method is simple, economical, accurate and precise , and could be successfully employed in the routine quality control for the simultaneous analysis of amlodipine besylate and telmisartan in tablets.

4.N.J.SHAH et al .,(2012)[25] have done Development and validation of a HPTLC method for the simultaneous estimation of telmisartan and hydrochlorothiazide in tablet dosage

forms. Its a simple, precise, accurate and rapid high performance thin layer chromatographic method has been developed and validated for the estimation of telmisartan and hydrochlorothiazide simultaneously in the combined dosage forms. The stationary phase used was precoated silica gel $60F_{254}$.The mobile phase used was the mixture of chloroform :methanol: toluene(2:5:5v/v/v).The detection of the spots was carried out at 272 nm. The method was validated in the terms of linearity, accuracy, precision and specificity .The calibration curve was found to be linear between 250 to 500ng/spot for telmisartan and 200 to 700 ng/spot for hydrochlorothiazide. The limit of the detection and the limit of quantification for the telmisartan were found to be 75 and 190ng/spot, respectively used to determine the drug content of the mareted formulation.

5.Ajit Pandey et al[26] .,(2011) have done UV- Spectrophotometric method for estimation of telmisartan in the bulk and tablet dosage form .A simple , precise and accurate UV spectrophotometric method has been developed and validated for the estimation of Telmisartan in the bulk and tablet dosage form. The zero order spectra of Telmisartan in 0.1 N NaOH shows λmax at 234.0 nm and the estimation was carried out by A(1% 1cm)and by comparison with standard. Calibration graph was found to be linear(r^2=0.999) over the concentration range 4-24µg/ml. The proposed method was validated for its accuracy, precision, specificity , ruggedness and the robustness .The method can be adopted in its routine analysis.

6.R.Vijayamirtharaj et al[27] .,(2010) have done Development and validation of the RP-HPLC method for the simultaneous estimation of telmisartan and atrovastatin calcium in tablet dosage forms. The present research deals with the development of RP-HPLC method for the determination of telmisartan and atrovastatin calcium in bulk and formulation using uv-detector. Selected mobile phase was a combination of acetoniltrile:buffer(0.001 potassium dihydrogen phosphate) 65:35 ph -4.0 (adjusted with orthophosphoric acid) and the wavelength selected was 250 nm. The flow rate was kept at 2.0 ml/min, and the injection volume was 10µl. Retention times of telmisartan and atrovastatin calcium was found to be 3.72 and 6.14 min respectively. Linearity of the method was found to be 319-480µg/ml for telmisartan and 86-130µg/ml for atrovastatin calcium.This method was validated according to ICH guidelines.

7.Patel Prashant B et al[28] .,(2012) have done Second order derivative spectrophotometric method for simultaneous the estimation of telmisartan and metoprolol in tablet dosage form

.Accurate, precise, rapid and economical method was developed for the estimation of telmisartan and metoprolol in bulk and tablet dosage form using second order derivative spectrophotometry. Wavelengths selected for quantitation were 299.5nm for telmisartan and 224nm for the metoprolol. Linearity was observed in the concentration range of 3-15µg/ml for both telmisartan and for metoprolol .The accuracy and precision were determined and found to comply with ICH guidelines.The proposed method was successfully applied for simultaneous estimation of both drugs in the commercial tablet preparation.

8.Zaveri Maitreyi et al[29] .,(2010) have done Development and validation of the RP-HPLC for the simultaneous estimation of atenolol and hydrochlorothiazide in the pharmaceutical dosage forms. The reverse phase high performance liquid chromatography method of atenolol and hydrochlorothiazide is individually available in USP-27 but no reference is available for combined estimation of atenelol and hydrochlorothiazide in tablet formulation. The aim of our present work was to develop a precise and validated RP-HPLC method for simultaneous determination of atenolol and hydrochlorothiazide in tablet formulation.the quantification was carried out by using Zorbax SB-CN column in isocratic mode with mobile phase, water :buffer: methanol(50:35:15).The flow rate was 1.2ml/min.The pea purity of atenolol and hydrochlorothiazide were 0.999 and 1.000 respectively.

9.Sushant K Shrivastava et al[30] .,(2012) have done Development and validation of a HPLC method for simultaneous estimation of amlodipine and telmisartan in the pharmaceutical dosage form. The chromatographic separation was achieved by using mobile phase acetonitrile and 0.05M sodium dihydrogen phosphate buffer(60:40) adjusted to ph-6.0,a c-18 column, perfectsil target ODS3.The mobile phase was pumped a flow rate of 0.8ml/min and eluents were monitored at 254nm.Retention times were 4.0 and 8.2 for amlodipine and telmisartan respectively. The method was validated in terms of accuracy, precision, linearity, range, specificity, limit of detection and limit of quantitation. The method was found to be efficient, accurate, precise, specific and economic and is suitable for routine quality control analysis.

10.Rekha Gangola et al[31] .,(2011) have done Spectrophotometric simultaneous determination of hydrochlorothiazide and telmisartan in the combined dosage form.Simple ,sensitive ,specific and economic spectrophotometric method was developed and validated for simultaneous quantitation of hydrochlorothiazide and telmisartan in tablet dosage form. New method based on the simultaneous estimation of drugs in a binary mixture without

previous separation was developed .In simultaneous equation method ,hydrochlorothiazide and telmisartan were quantified using their absorptivity values of at selected wavelengths, via., 273 nm and 295 nm respectively. The accuracy and reproducibility of the proposed method was statistically validated by recovery studies. The simultaneous equation method permits simple, rapid and direct determination of hydrochlorothiazide and telmisartan in commercially available tablet dosage form.

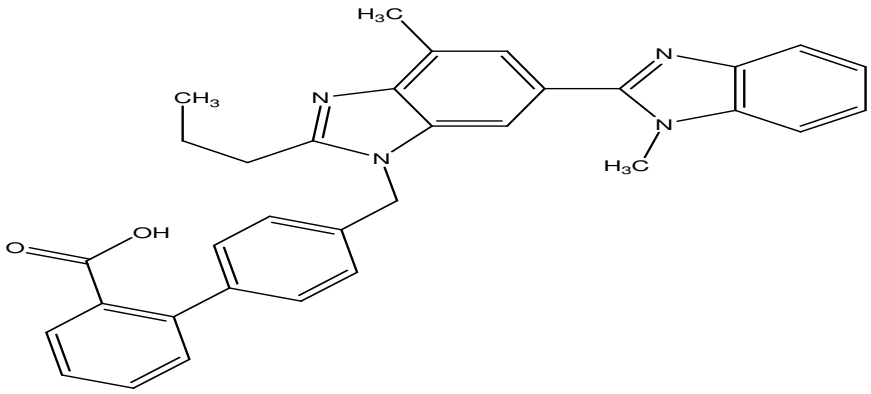
11.Devanand B.Shinde et al[32] ., (2011) have done stability indicating LC method for simultaneous estimation of Telmisartan and Hydrochlorothiazide in dosage form. A simple ,rapid and precise method is developed for the quantitative simultaneous estimation of Telmisartan and Hydrochlorothiazide in combined pharmaceutical dosage form. A chromatographic separation of the two drugs was achieved with an ACE 5 C_{18} (250 × 4.6 mm)analytical column using buffer : acetonitrile (55:45v/v).The buffer used in the mobile phase containes 0.1M sodium perchlorate monohydrate in double distilled water and pH 3.0 was adjusted with trifloro acetic acid .The instrument settings were flow rate 1.5ml/min ,column temperature at30˚c.The retention times for Hydrochlorothiazide and telmisartan were 3.17 and 11.26.The RSD for both drugs were less than 2%.The proposed method was found to be suitable and accurate for quantitative determination.

12. Joshi Priyanka et al[33] .,(2011) have done Development and validation of reverse phase HPLC method for simultaneous of Hydrochlorothiazide and Telmisartan in tablet dosage form. Present work describes a simple, accurate, precise and reproducible reverse phase High Performance Liquid Chromatographic method for simultaneous estimation of Hydrochlorothiazide and Telmisartan in tablet dosage form on RP C-18 Column (Hypersil Gold, 25cm x 4.6mm, 5µm) using Acetonitrile and buffer (0.05M KH2PO4, pH 3.0±0.02, 35:65 v/v) as mobile phase at a flow rate of 1.0 ml/min and the detection wavelength was 225nm. The retention time for Hydrochlorothiazide and Telmisartan was found to be 3.71 and 11.72 min. respectively. Detection response for both Hydrochlorothiazide and Telmisartan were found to be linear low in concentration range of 6-22.4mcg/ml and 24.12-56.27mcg/ml respectively in the linearity study, regression equation and coefficient of correlation for Hydrochlorothiazide and Telmisartan were found to be (y = 33409x + 5518, r2 =0.9999) and (y = 111545x – 5850.4, r2 = 0.9999). Proposed method was validated for accuracy, precision, linearity, range, ruggedness & robustness.

2.2. DRUG PROFILE

2.2.1 TELMISARTAN[34]:

Molecular Structure :



Telmisartan

Molecular Formula : $C_{33}H_{30}N_4O_2$

Chemical Name : 2-(4-{[4-methyl-6-(1-methyl-1H-1,3-benzodiazol-2-yl)-2-propyl-1H-1,3-benzodiazol-1-yl]methyl}phenyl)benzoic acid

Molecular Weight : 514.62 g/mol

Category : Anti hypertensive agents

Angiotensin II antagonists

Physical Properties of Telmisartan

Color **:** White to slight yellowish powder

Melting Point **:** 261-263^{o}C

State **:** Solid

Solubility **:** Practically insoluble in water ,sparingly soluble in strong acid and strong base

Pharmacological Properties

Dosage Form **:** Tablet

Route of administration **:** Oral

Pharmacology **:** Telmisartan was rapidly absorbed after oral administration in Hypertensive patients. Absolute bioavailability in adults was 42-100% for the tablet.

Clinical pharmacology:

Mechanism of Action [35] **:** Telmisartan interferes with the binding angiotensin II to the angiotensin AT_1 –receptor by binding reversibly and selectively to the receptors in vascular smooth muscle and the adrenal gland.A angiotensin II is a vasoconstrictor ,which also stimulates the synthesis and release of aldosterone ,blockage of its effects results in decreases in systemic vascular resistance.

Telmisartan acts as a selective modulator of peroxisome proliferator activated receptor-γ ,a regulator of insulin and glucose metabolism. It is believed that telmisartan's dual mode of action may provide protective benefits against the vascular and renal damage caused by diabetis and cardiovascular disease.

Absorption and distribution:

Following oral administration, peak concentrations (Cmax) of telmisartan are reached in 0.5-1 hour after dosing. Food slightly reduces the bioavailability of telmisartan, with a reduction in the area under the plasma concentration-time curve (AUC) of about 6% with the 40 mg tablet and about 20% after a 160 mg dose. The absolute bioavailability of telmisartan is dose dependent. At 40 and 160 mg the bioavailability was 42% and 58%, respectively. The pharmacokinetics of orally administered telmisartan are nonlinear over the dose range 20-160 mg, with greater than proportional increases of plasma concentrations (Cmax and AUC) with increasing doses. Telmisartan shows bi-exponential decay kinetics with a terminal elimination half life of approximately 24 hours. Trough plasma concentrations of telmisartan with once daily dosing are about 10-25% of peak plasma concentrations. Telmisartan has an accumulation index in plasma of 1.5 to 2.0 upon repeated once daily dosing.

Telmisartan is highly bound to plasma proteins (>99.5%), mainly albumin and α1 - acid glycoprotein. Plasma protein binding is constant over the concentration range achieved with recommended doses. The volume of distribution for telmisartan is approximately 500 liters indicating additional tissue binding.

Metabolism and Elimination:

Following either intravenous or oral administration of 14C-labeled telmisartan, most of the administered dose (>97%) was eliminated unchanged in feces via biliary excretion; only minute amounts were found in the urine (0.91% and 0.49% of total radioactivity, respectively).

Telmisartan is metabolized by conjugation to form a pharmacologically inactive acylglucuronide; the glucuronide of the parent compound is the only metabolite that has been identified in human plasma and urine. After a single dose, the glucuronide represents approximately 11% of the measured radioactivity in plasma. The cytochrome P450 isoenzymes are not involved in the metabolism of telmisartan.

Total plasma clearance of telmisartan is >800 mL/min. Terminal half-life and total clearance appear to be independent of dose.

Pharmacodynamics

In normal volunteers, a dose of telmisartan 80 mg inhibited the pressor response to an intravenous infusion of angiotensin II by about 90% at peak plasma concentrations with approximately 40% inhibition persisting for 24 hours.

Interactions :

Digoxin : When telmisartan was coadministered with digoxin, median increases in digoxin peak plasma concentration (49%) and in trough concentration (20%) were observed. It is, therefore, recommended that digoxin levels be monitored when initiating, adjusting, and discontinuing telmisartan to avoid possible over- or under-digitalization.

Warfarin : Telmisartan administered for 10 days slightly decreased the mean warfarin trough plasma concentration; this decrease did not result in a change in International Normalized Ratio (INR).

Indications:

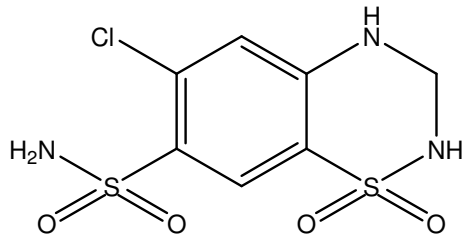
Telmisartan is indicated for the treatment of hypertension. It may be used alone or in combination with other antihypertensive agents.

Storage:

Store at 25°C (77°F); excursions permitted to 15°-30°C (59°-86°F) [see USP Controlled Room Temperature]. Tablets should not be removed from blisters until immediately before administration.

2.2.2 HYDROCHLOROTHIAZIDE[36]:

Molecular Structure:



Hydrochlorothiazide

Molecular Formula : $C_7H_8ClN_3O_4S_2$

Chemical Name : 6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzthiadiazine-7-sulfonamide

Molecular Weight : 297.74 g/mol

Category : Thiazide diuretic

Anti hypertensive agent

Na-Cl symporter inhibitors

Physical Properties of Hydrochlorothiazide

Color : White to half white crystalline powder

Melting Point : 273-275^{o}C

State : Solid

Solubility : Soluble in dilute ammonia and NaOH ,also soluble in methanol, acetone, ethanol . Freely soluble in NaOH ,n-butylamine , dimethylformamide.

Pharmacological Properties

Dosage Form : Tablet

Route of administration : Oral

Pharmacology :Hydrochlorothiazide variably absorbed from GI tract.Bioavailability of drug is 70%.Does not undergo significant metabolism(>95% of drug unchanged in urine)

Clinical pharmacology:

Mechanism of Action[37,38] : Hydrochlorothiazide, is a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the Na-Cl Symporter in the distal convoluted tubule, which was responcible for 5% of sodium reabsorption. Normally, the Na-Cl symporter transports Na and Cl from the lumen into the epithelial cell lining distal convoluted tubule. The energy for this is provided by a Na gradient established by Na-k ATPases on the basolateral membrane. Once Na was entered in the cell, it is transported out in the basolateral interstitium via Na-k ATPase, causing an increase in the osmolarity of the interstitium, there by establishing an osmatic gradient for water reabsorption. By blocking the Na-Cl symporter, hydrochlorothiazide effectively reduces the osmatic gradient and water reabsorption throughtout the nephron.Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the Na-Cl in the distal convoluted , which is responsible for 5% of total sodium reabsorption. Normally, the Na-Cl symporter transports Na and Cl from the lumen in the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a Na gradient established by Na-K ATPases on the basolateral membrane. Once Na has entered the cell, it is transported out into the basolateral interstitium

via the Na-k ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron.

Pharmacodynamics:

In normal volunteers, a dose of Hydrochlorothiazide 25 mg inhibited the pressor response to an intravenous infusion of angiotensin II by about 90% at peak plasma concentrations with approximately 40% inhibition persisting for 24 hours.

Indications and Usage：

Hydrochlorothiazide tablets are indicated as adjunctive therapy in edema associated with congestive heart failure, hepatic cirrhosis, and corticosteroid and estrogen therapy.

Hydrochlorothiazide tablets have also been found useful in edema due to various forms of renal dysfunction such as nephrotic syndrome, acute glomerulonephritis, and chronic renal failure.

Hydrochlorothiazide tablets are indicated in the management of hypertension either as the sole therapeutic agent or to enhance the effectiveness of other antihypertensive drugs in the more severe forms of hypertension.

Drug Interactions:

When given concurrently the following drugs may interact with thiazide diuretics. Alcohol, barbiturates, or narcotics—potentiation of orthostatic hypotension may occur.Antidiabetic drugs (oral agents and insulin)—dosage adjustment of the antidiabetic drug may be required. Other antihypertensive drugs—additive effect or potentiation.

3. AIM AND PLAN OF WORK

AIM :

- Telmisartan is a angiotensin-II inhibitor. It is used in the treatment of hypertension. Hydrochlorothiazide is a thiazide diuretic. It is also used in the treatment of hypertension. The combination of these two drugs used effectively in the treatment of hypertension in combined dosage form.
- Literature survey reveals that Telmisartan and Hydrochlorothiazide is estimated individually and simultaneously by UV, derivative – HPLC, Plasma RP-HPLC and Plasma LC/MS/MS methods. Few RP-HPLC methods were reported for estimation of Telmisartan, Ramipril and amlodipine in pharmaceutical formulation.

- RP-HPLC, LC-MS/MS and HPTLC methods were reported for the simultaneous estimation of Metoprolol and hydrochlorothiazide in human plasma and in formulations. Also UV, HPLC, LC–MS, HPTLC and enzymatic assay methods were reported for the simultaneous estimation of Telmisartan with other anti-hypertensive drugs .

- Thus, efforts were made to develop economical and sensitive analytical method for Simultaneous estimation of Telmisartan and Hydrochlorothiazide in their combined dosage form.

Plan of work :

The plan of the proposed work includes the following steps:

- The extensive survey of literature for Telmisartan and Hydrochlorothiazide regarding their physico-chemical properties and analytical methods. This forms the basis for the development of methods.
- Selection of suitable solvent for quantitative extraction of analyte present in the formulations. The solvent should be readily available, economical and of analytical grade.

- To develop initial chromatographic conditions by selection of suitable column and appropriate wavelength in UV for detection and optimization of the method.
- To validate analytical method developed as per the ICH Q2B guidelines.

4. EXPERIMENTAL PART

4.1 MATERIALS AND INSTRUMENTS

4.1.1 Instruments:

Table No 3: Table showing list of the Instruments used

S. No	Name of the Instrument	Make	Model
1.	HPLC	Water	Alliance-2695 PDA Waters-2996
2.	Electronic balance	Shimadzu	AY 220
3.	Digital pH meter	Digisun Electronics	7007
4.	Centrifuge	Thermolab	R_8C
5.	PhotoStability Chamber	Thermolab	943/03/0607

4.1.2 Reagents and chemicals:

Table No 4: Table showing list of the chemicals used

S. No	Name	Grade	Manufacturer/ Supplier
1.	Telmisartan and Hydrochlorothiazide working standard	-	
2.	Acetonitrile	HPLC	Merck
3.	Trifluoro acetic acid	HPLC	Merck
4.	Methanol	HPLC	Merck
5.	Water	-	-
6.	Milli Q Water	HPLC	-
7.	Potassium dihydrogen phosphate buffer	HPLC	-

4.2 METHOD DEVELOPMENT AND OPTIMIZATION OF TELMISARTAN AND HYDROCHLOROTHIZIDE

1. SELECTION OF DETECTOR WAVELENGTH:

The wave length selection is made at 271 nm since all the two compounds maximum absorbance in UV spectrum as reported in the literature is in 271 nm.

4.2.2 OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

a. Selection of Stationary phase:

As the drugs were polar in nature, non- polar stationary phase was preffered.The hypersil BDS column was selected as reported in the literature.

b.Selection of mobile phase:

The hydrochlorothiazide soluble in acetonitrile,methanol,water.But Telmisartan only soluble in 50:50 acetonitrile and methanol.So the buffer methanol and acetonitrile were selected as starting mobile phases.The starting buffer was potassium dihydrogen phosphate buffer because the pH of this buffer is related to pk_a values of both drugs.

b.Procedure:

Preparation of mobile phase:

About 3.2 g of potassium dihydrogen phosphate was weighed and dissolved in 1000 ml water.The pH was adjusted to 3.4 with ortho phosphoric acid.500 ml of this buffer solution was added to 250 ml of acetonitrile and to this the 250 ml of methanol was added to produse 1000 ml. Mobile phase buffer : acetonitrilr:methanol(50:25:25).

Preparation of Standard Solution:

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed and transferred into 100ml volumetric flask, about 70ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 5 ml of solution was pipette out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.Inject 20 µl of the standard solution into the

chromatographic system and measure the area for the Telmisartan and Hydrochlorothiazide peaks and calculate the %Assay by using the assay formula.

4.3 .TRIAL&ERROR METHODS:

TRIAL- 1:

CHROMATOGRAPHIC CONDITIONS:

Column : Hypersil BDS (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 271nm

Flow rate : 1ml/min

Injection volume : 20µl

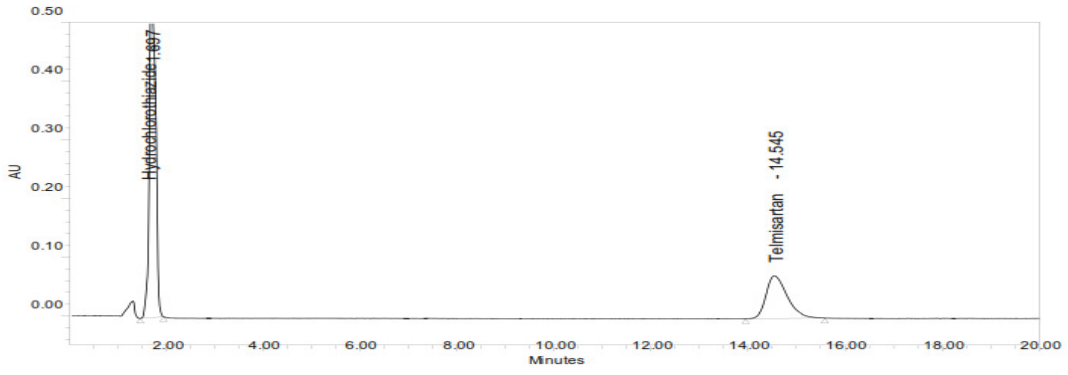
Run time : 10min

Mobile phase : Buffer (phosphate buffer): Methanol:ACN (50:25:25)

P_H :3.4

Results of Trial-1: Telmisartan Peak was not sharp.USP plate count was recorded as 889.Due to asymmetry in peaks,identification of interferences and USP plate count not meet the acceptance criteria . so reduce RT's another trial is made with change in mobile phase-buffer pH and flow rate.

Fig No: 4.1



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorothiazid	1.697	5107347	69.81	581258	1.1	889
2	Telmisartan	14.545	2208722	30.19	72922	1.4	5735

TRIAL-2:

CHROMATOGRAPHIC CONDITIONS:

Column : Hypersil BDS (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 271nm

Flow rate : 1.2ml/min

Injection volume : 20µl

Run time : 10min

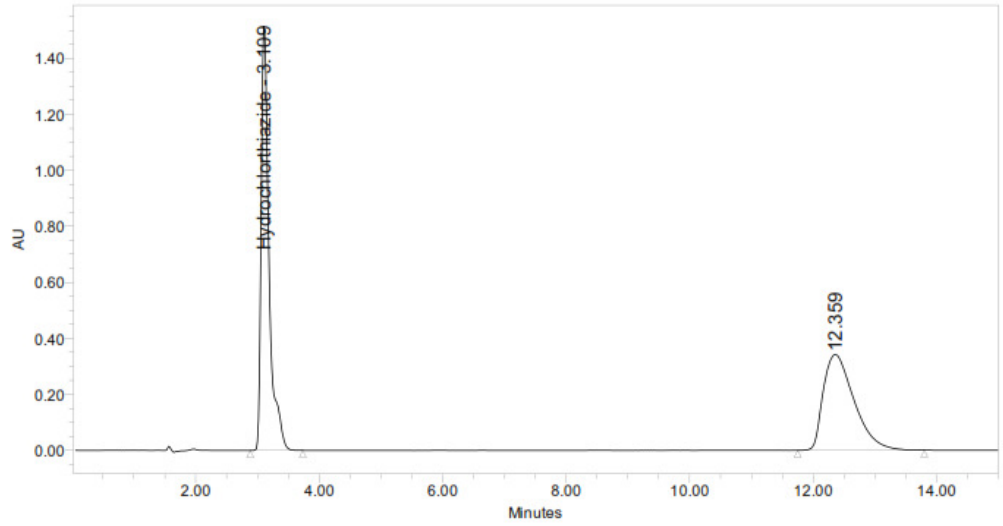
Column temperature : 30°c

Mobile phase : Buffer (PHOSPHATE BUFFER): ACN (50:50)

P_H : 3.4

Results of Trial-2: Retention time observed at 3.109 and 12.359. Due to asymmetry in peaks another trail is made inorder to reduce RT's with change in mobile phase.

Fig No: 4.2



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorothiazide	3.109	2346402	69.81	581258	1.9	1983
2	Telmisartan	12.349	1183838	30.19	72922	2.0	1847

TRIAL-3:

CHROMATOGRAPHIC CONDITIONS:

Column : Hypersil BDS (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 271nm

Flow rate : 0.8ml/min

Injection volume : 20µl

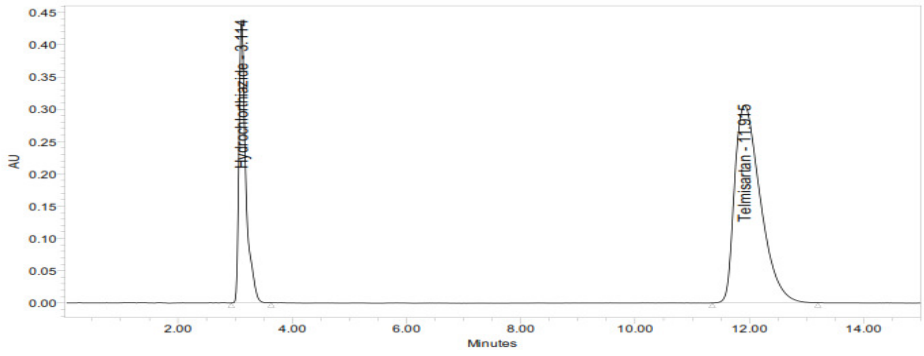
Run time : 10min

Column temperature : 30°c

Mobile phase : Buffer (Ammonium dihydrogen phosphate buffer): ACN (50:50)

Results of Trial-3: RT's were observed at 3.114(Hydrochlorothiazide) and 11.915(Telmisartan). Inorder to reduse the RT'S another trial is made with change in mobile phase.

FIG:4.3



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	3.114	3630667	27.50	437314	1.8	3887
2	Telmisartan	11.915	9572407	72.50	305851	1.6	3378

TRIAL-4

CHROMTOGRAPHIC CONDITIONS:

Column : Symmetry C_{18} (INERTSIL ODS 150x4.6mm)

Detector : 271

Flow rate : 1ml/min

Injection volume : 20µl

Run time : 12 min

Column temperature : 25˚C

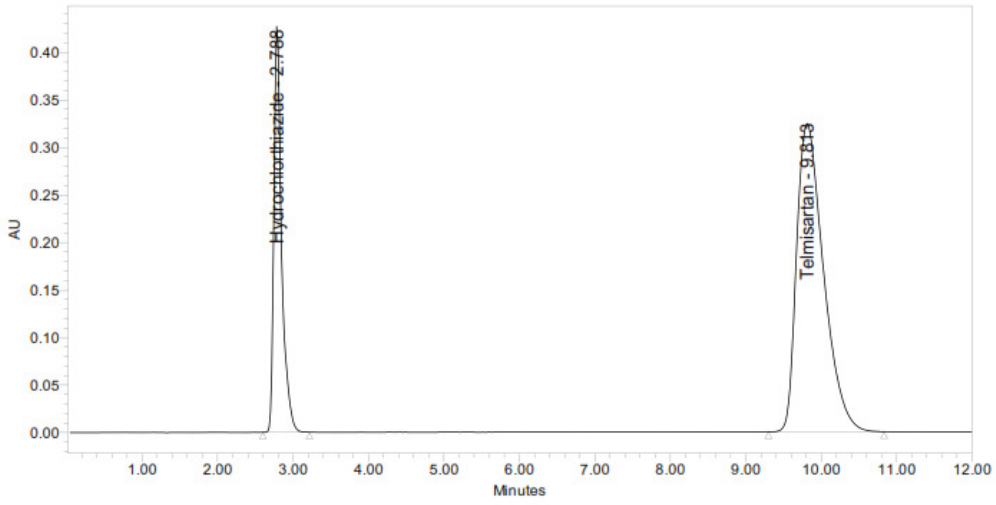
Mobile phase :Trifluoro acetic acid:ACN(70:30)

Preparation of mobile phase:

Mix a mixture of above buffer 700mL (70%) and 300 mL of ACN (30%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vaccum filtration.

Results of Trial-4 : Retention times were observed at 2.788(Hydrochlorothiazide) and 9.813(Telmisartan).The peaks were sharp with less tailing and USP plate count meet the acceptance criteria and RT'S were reduced .Hence the trial-4 is optimized for analysis.

FIG:4.4



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.788	3027459	27.40	426268	1.6	4038
2	Telmisartan	9.813	8022156	72.60	324417	1.5	3554

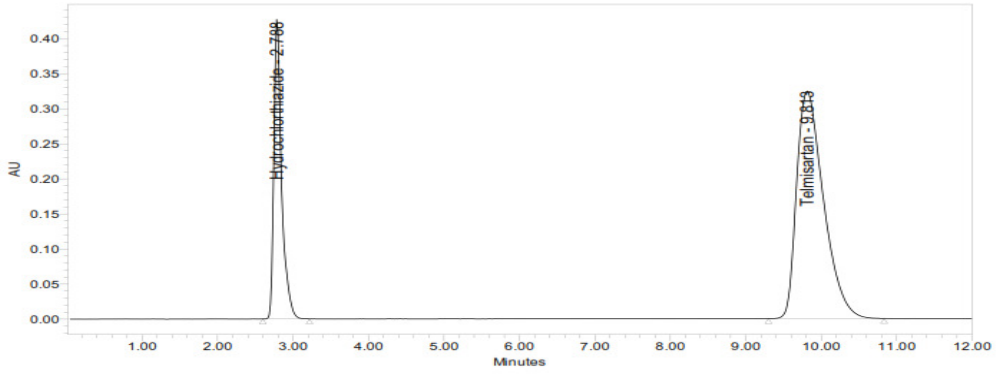
4.4. ANALYTICAL METHOD-OPTIMISATION

Aim: The present study is to develop a new reverse phase liquid chromatographic method for simultaneous determination of Telmisartan and Hydrochlorothiazide in tablet dosage form.

Table No : 5 Table showing Optimized Chromatographic Parameters

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	isocratic elution
Mobile phase	Solvent-A: Trifluoro acetic acid pH-3 Solvent-B: Acetonitrile(70:30)
Column	INERTSIL ODS(4.6 x 150mm, 5 µm)
Flow rate	1 mL/ min
Detection Wavelength	271 nm
Injection volume	20 µl
Column oven temperature	Ambient(25°C)
Run time	12 min

Fig No: 4.5 Chromatogram showing peaks of standard solution of Telmisartan and Hydrochlorothiazide



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.788	3027459	27.40	426268	1.6	4038
2	Telmisartan	9.813	8022156	72.60	324417	1.5	3554

4.5 QUANTITATIVE DETERMINATION OF THE DRUG BY USING THE DEVELOPED METHOD

Sample : Telmisartan and Hydrochlorothiazide

Label claim : 40mg,12.5mg

Standard Solution Telmisartan and Hydrochlorothiazide:

Accurately 80 mg of Telmisartan and 25mg of Hydrochlorothiazide were weighed and transferred into 100ml volumetric flask, about 70ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 5ml of solution was pipette out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of the standard solution into the chromatographic system and measure the area for the Telmisartan and Hydrochlorothiazide peaks and calculate the %Assay by using the assay formula.

Sample Solution Telmisartan and Hydrochlorothiazide:

20 Telmisartan and Hydrochlorothiazide tablets were weighed and the average weight was calculated. Accurately the sample equivalent to 80 mg of Telmisartan &25 mg of Hydrochlorothiazide was weighed & transferred into 100ml volumetric flask about 70ml of diluent was added and sonicated for 5 minutes to dissolve it content. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (stock solution).

5ml of stock solution was pipetted out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Assay formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \text{average weight of tablet}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

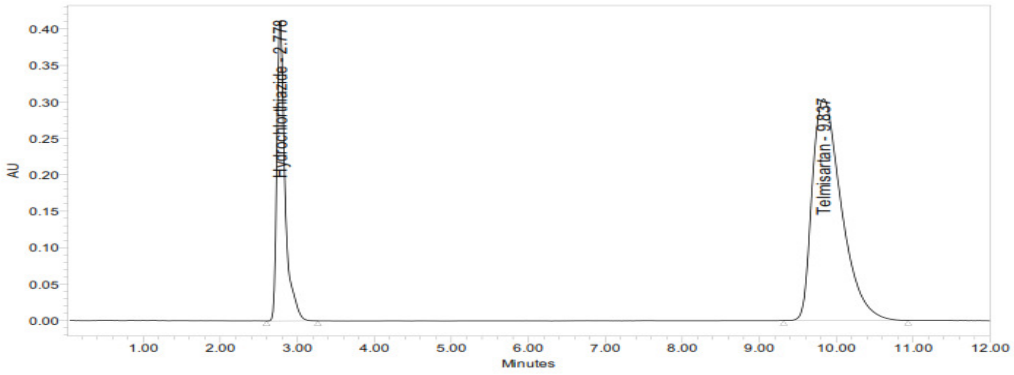
DT = Dilution of sample solution

Acceptance criteria: The limit of assay is in between the 98% - 102%

The chromatograms are as shown in Fig No: 4.6 & 4.7 and assay results are tabulated and are as shown in Table No: 6

Chromatogram showing peaks of standard solution of Telmisartan and Hydrochlorothiazide.

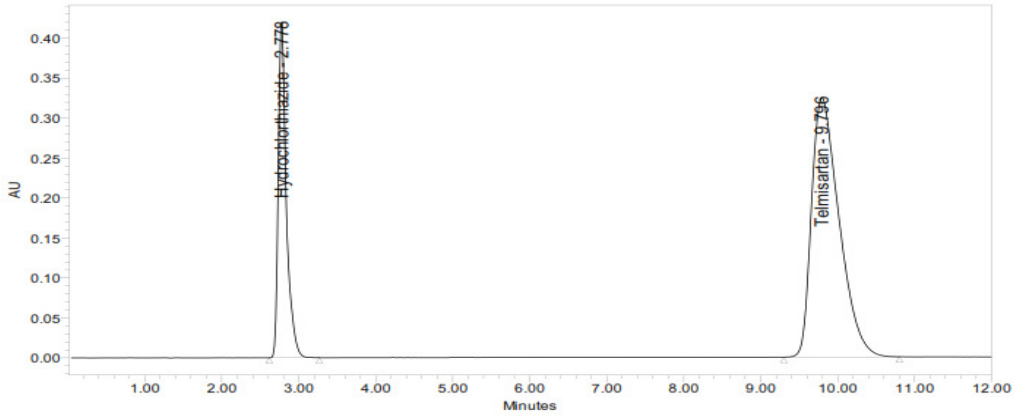
Fig No :4.6



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.778	3028837	27.43	412784	1.7	3953
2	Telmisartan	9.837	8013447	72.57	304186	1.6	3312

Chromatogram showing peaks of test solution of Telmisartan and Hydrochlorothiazide.

Fig No : 4.7



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.778	3048427	27.41	421187	1.5	3888
2	Telmisartan	9.796	8073243	72.59	325061	1.5	3349

Table showing Assay Results of Telmisartan and Hydrochlorothiazide:

Table No: 6

S. No	Compound name	Assay value
1.	Telmisartan	99.56%
2.	Hydrochlorothiazide	99.1%

4.6. VALIDATION

Definition: Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below.

- Accuracy
- Precision
- Specificity
- Linearity & Range
- Robustness
- Ruggedness
- System suitability testing

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

4.6.1 SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated and the parameters like tailing factor, retention

time, theoretical plates per unit, resolution factor are determined and the results are tabulated and are as shown in Table No: 7

Method

Preparation of Standard solution:

Standard Solution Telmisartan and Hydrochlorothiazide:

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent (ACN:MET)was added to two flasks and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions were filtered through 0.45µm membrane filter (Stock solutions).

From above solutions 5 ml Telmisartan stock solution and 5ml of Hydrochlorothiazide were pipetted out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

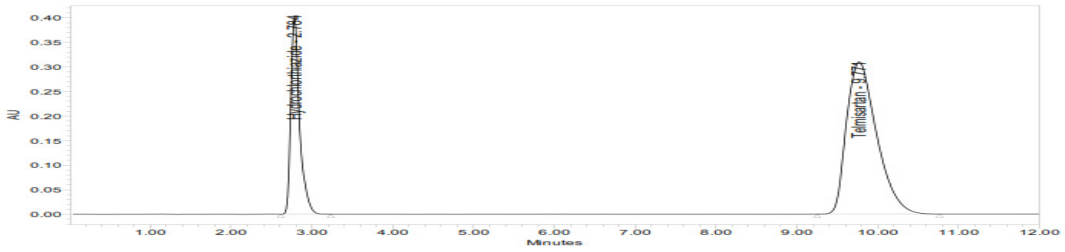
Inject 20 µl of the standard solution into the chromatographic system and measure the area for the Telmisartan and Hydrochlorothiazide peaks and calculate the %Assay by using the assay formula.

Acceptance criteria:

a. The column efficiency is not less than 2000 theoretical plates.
b. The tailing factor for the analyte peak is not more then 2.0.
c. The relative standard deviation for the replicate injections more than 2.0%.

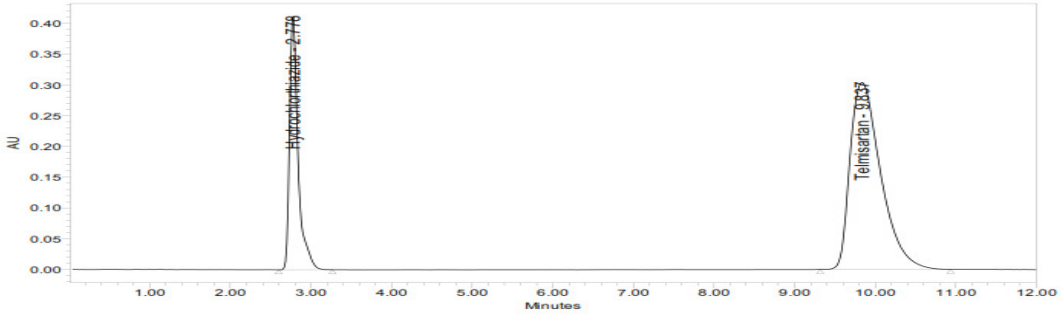
Chromatograms showing system suitability testing of Standard Solutions of Telmisartan and Hydrochlorothiazide.

Fig No : 4.8



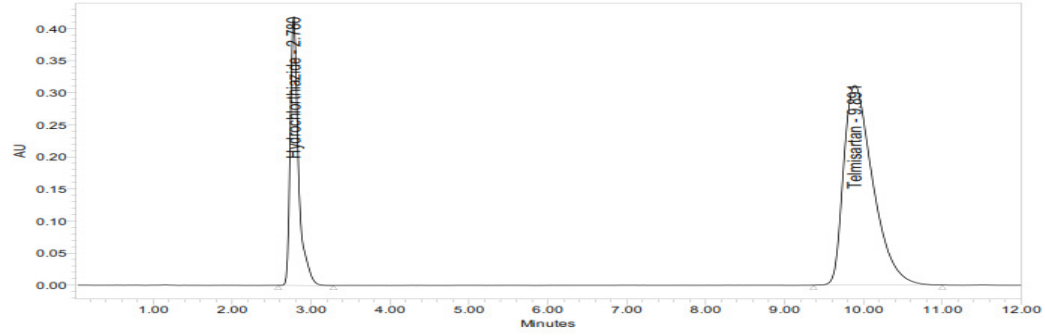
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.784	3022485	27.36	402996	1.5	3666
2	Telmisartan	9.771	8026557	72.64	310471	1.4	3138

Fig No : 4.9



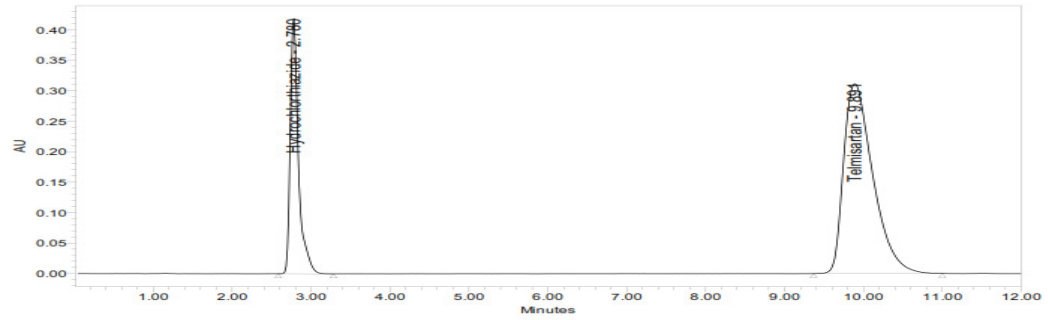
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.778	3028837	27.43	412784	1.7	3953
2	Telmisartan	9.837	8013447	72.57	304186	1.6	3312

Fig NO:4.10



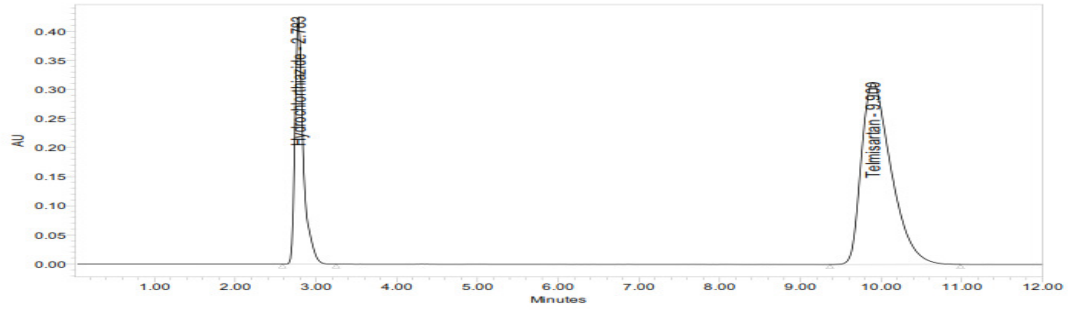
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.780	3018824	27.38	418189	1.6	4108
2	Telmisartan	9.891	8007693	72.62	311578	1.6	3418

Fig NO :4.11



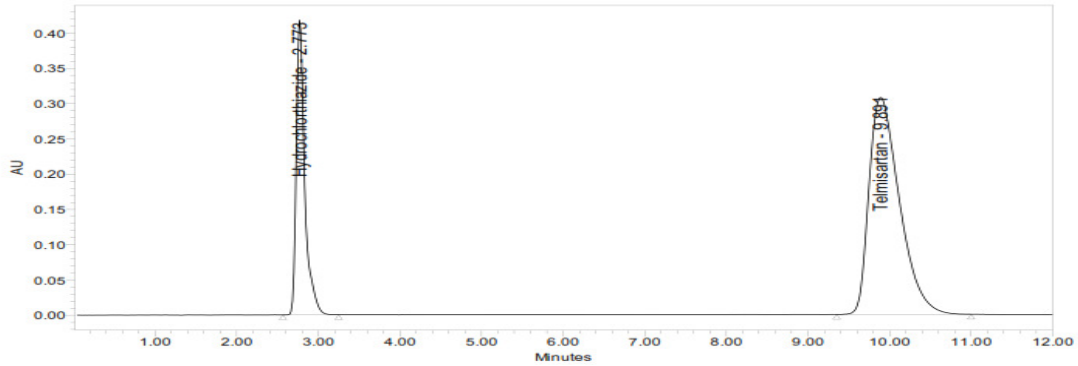
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.780	3018824	27.38	418189	1.6	4108
2	Telmisartan	9.891	8007693	72.62	311578	1.6	3418

Fig No: 4.12



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.783	3022719	27.40	422399	1.6	4219
2	Telmisartan	9.900	8008710	72.60	312851	1.5	3368

Fig No : 4.13



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.773	3004344	27.40	418971	1.6	3930
2	Telmisartan	9.891	7960524	72.60	307988	1.5	3312

Table showing list of system suitability parameters of Telmisartan and Hydrochlorothiazide.

Table No : 7

Parameters	**Telmisartan**	**Hydrochlorothiazide**
Tailing factor	1.5	1.6
Retention time	2.778	9.831
Theoretical plates per unit	2766.24	2853.19

4.6.2 ACCURACY

The accuracy of an analytical method is the closeness of that results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte.

Determination:

The accuracy of the analytical method was determined by applying the method to the analyzed samples, to which known amounts of analyte had been added. The accuracy was calculated from the test results as the percentage of analyte recovered by the assay.

Procedure:

Preparation of Standard solution :

Accurately 80 mg of Telmisartan and 25 mg Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent was added to each flask and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions was filtered through 0.45µm membrane filter (Stock solutions).

From these above solutions 5ml of Telmisartan and 5 ml of Hydrochlorothiazide stock solutions was pipetted out and transferred into 50ml of

volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 50% sample solution(40µg/ml Telmisartan and 12.5µg/ml Hydrochlorothiazide):

Accurately weighed quantity of placebo and transferred into two 100 ml flasks.To this flask accurately weighed quantity of 40 mg of Telmisartan and 12.5 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent was added to each flask and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solutions).

From these above solutions 5ml of Telmisartan and 5ml of Hydrochlorothiazide solutions were pipetted out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 100% solution(80µg/ml Telmisartan and 25µg/ml Hydrochlorothiazide):

Accurately weighed quantity of placebo transferred into two 100 ml flasks.To this 80 mg of Telmisartan and 25 mg Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent was added to each flask and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions was filtered through 0.45µm membrane filter (Stock solutions).

From these above solutions 5ml of Telmisartan and 5ml of Hydrochlorothiazide solutions were pipetted out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Preparation of 150% solution(120 µg/ml Telmisartan and 37.5µg/ml Hydrochlorothiazide):

Accurately weighed quantity of placebo transferred into two 100 ml flasks.To this accurately weighed quantity of 120 mg of Telmisartan & 37.5 mg Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric

flasks, about 70ml of diluent was added to each flask and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions was filtered through 0.45µm membrane filter (Stock solutions).

From these above solutions 5ml of Telmisartan and 5ml of Hydrochlorothiazide solutions were pipetted out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of placebo and standard solutions of Accuracy -50%, Accuracy -100% and Accuracy -150% solutions 3 times into HPLC. Now calculate the amount obtained and amount added (API) for Telmisartan and Hydrochlorothiazide samples.

Calculate the concentration in µg/ml in the spiked placebo in all the above cases by comparing with the standard solution. Calculate the individual recovery and mean recovery values. The chromatograms are as shown in Fig. No: 4.14 to 4.22 and the results are tabulated and shown in Table No: 8

Formula:

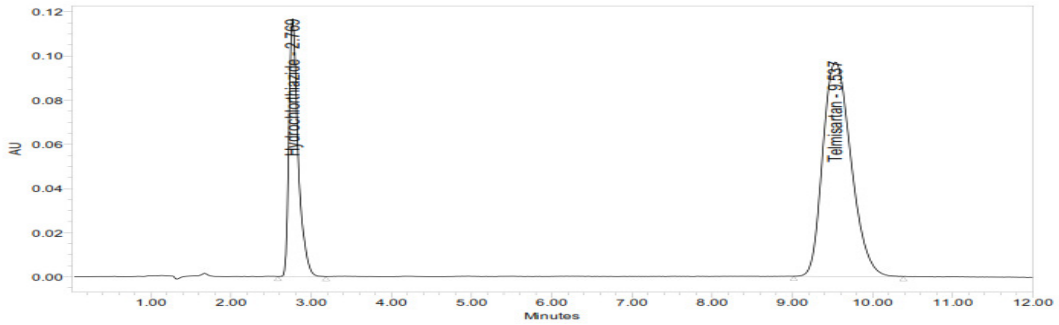
$$\%\text{recovery} = \frac{\text{(Amount recovered)}}{\text{(Actual amount added)}} \times 100$$

Acceptance criteria:

Percentage recovery in all the cases should be between 98.0 and 102.0 %.

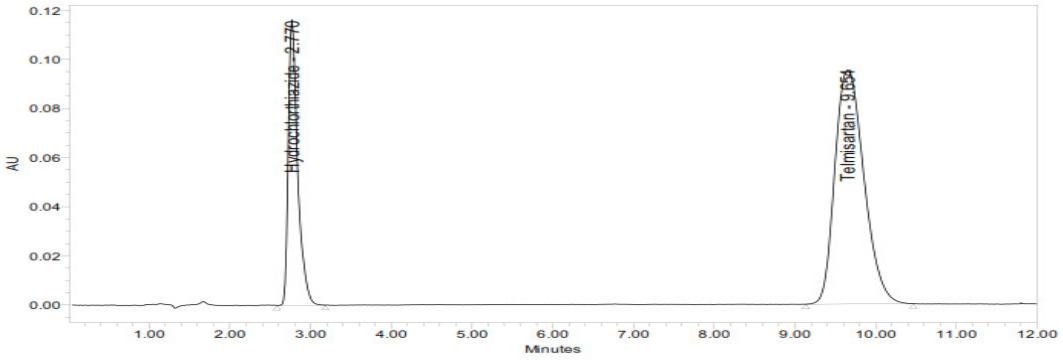
Chromatograms showing Accuracy 50% of Telmisartan and Hydrochlorothiazide.

Fig No : 4.14



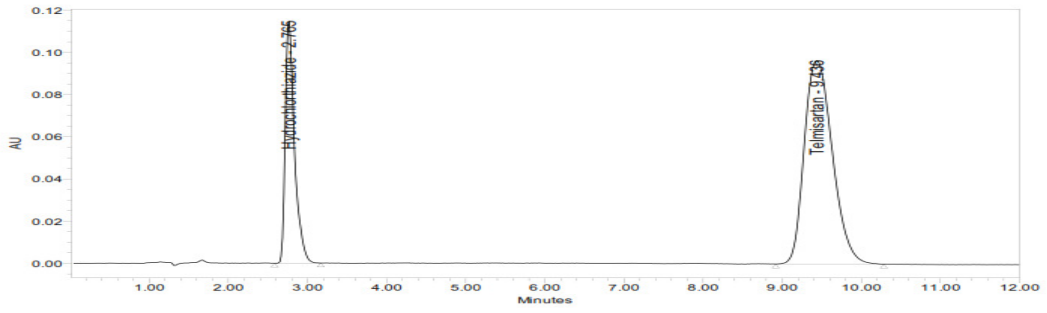
	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.769	924988	28.02	116663
2	Telmisartan	9.537	2375643	71.98	97136

Fig NO: 4.15



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.770	933665	28.17	116384
2	Telmisartan	9.654	2380181	71.83	95374

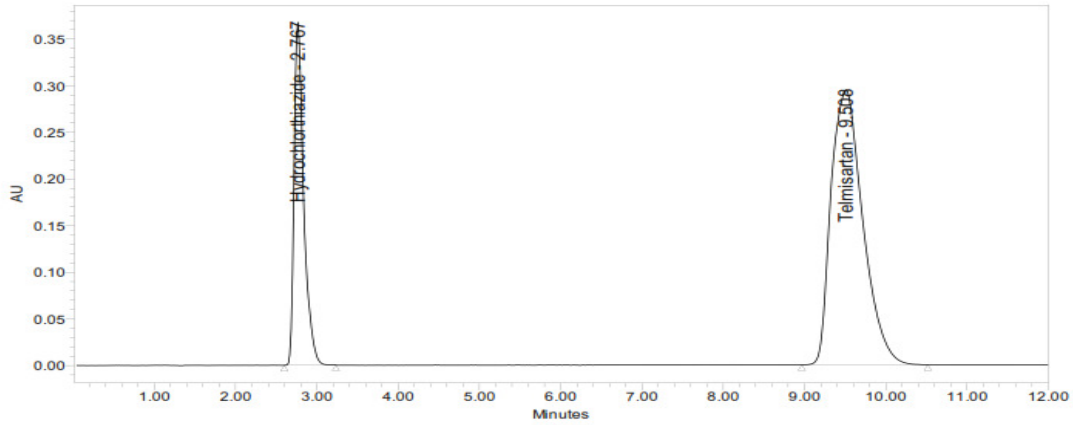
Fig NO :4.16



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.765	927877	28.00	114601
2	Telmisartan	9.436	2385885	72.00	96526

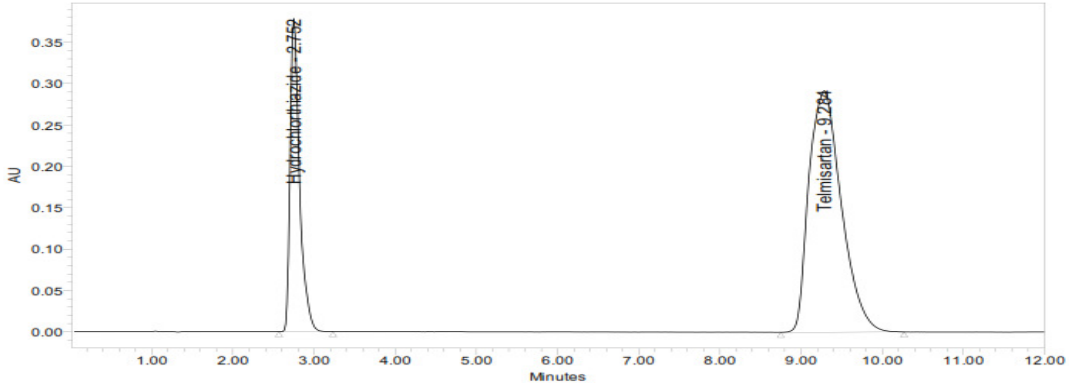
Chromatograms showing Accuracy -100% of Telmisartan and Hydrochlorothiazide.

Fig No :4.17



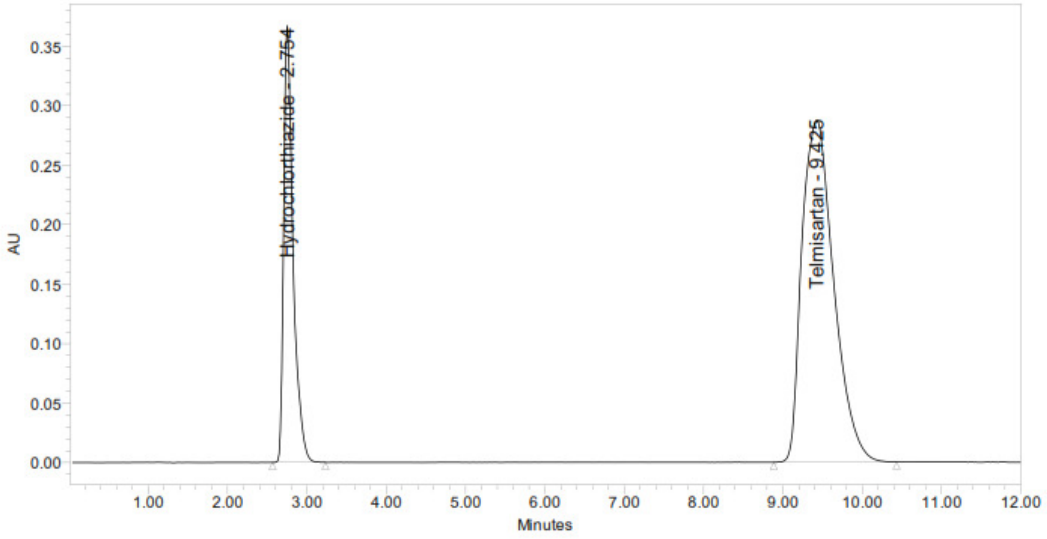
	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.767	3017884	27.41	367239
2	Telmisartan	9.508	7990697	72.59	295192

FigNO:4.18



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.752	3058022	27.41	378323
2	Telmisartan	9.284	8098133	72.59	291830

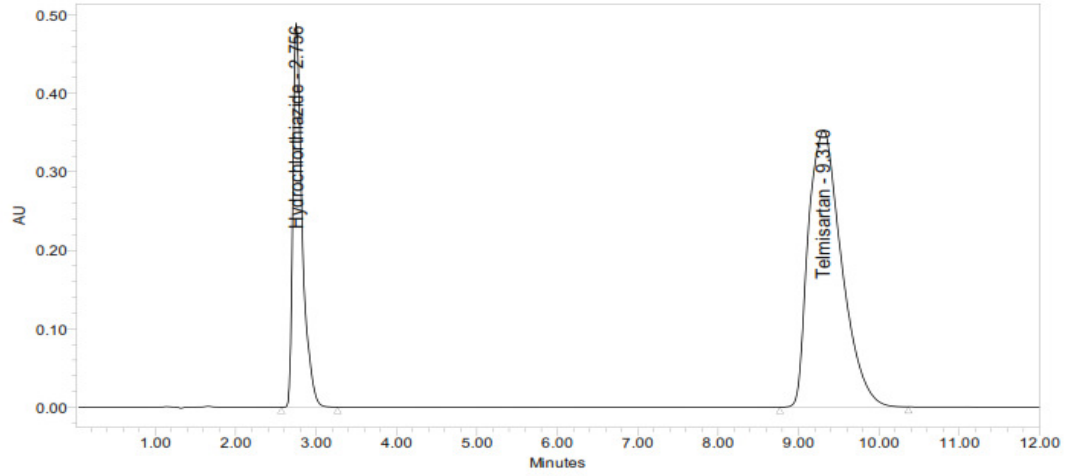
Fig NO:4.19



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.754	3019049	27.41	367441
2	Telmisartan	9.425	7993509	72.59	287599

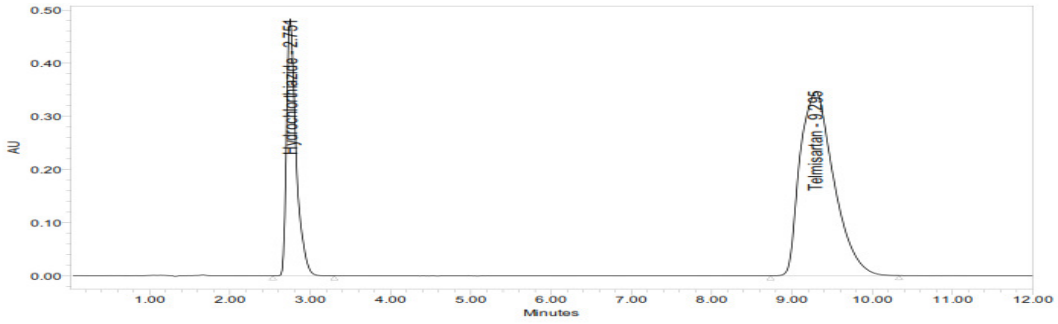
Chromatogram showing Accuracy – 150% of Telmisartan and Hydrochlorothiazide.

Fig No : 4.20



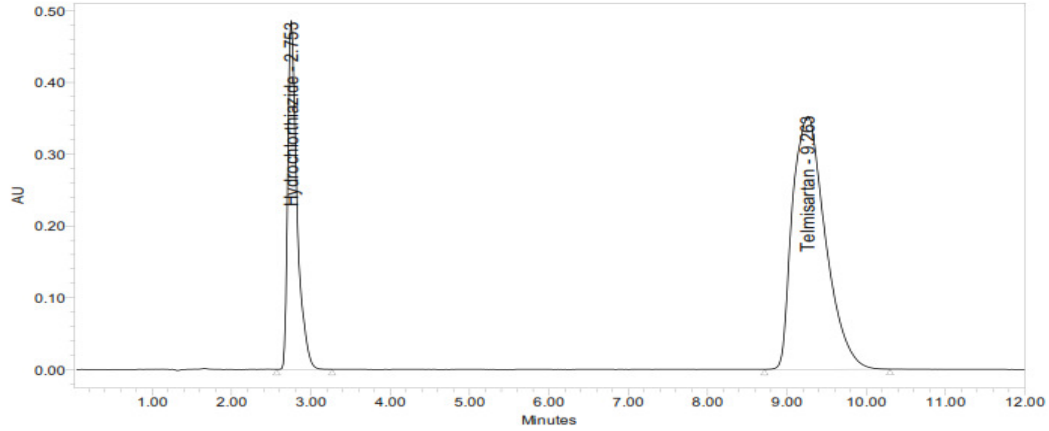
	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.756	3969777	28.19	491876
2	Telmisartan	9.310	10110923	71.81	352887

FigNO: 4.21



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.751	3992816	28.29	483857
2	Telmisartan	9.295	10122823	71.71	347419

FigNO:4.21



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.753	3941891	27.99	485834
2	Telmisartan	9.263	10142375	72.01	352539

Table showing results of % Recovery studies for Telmisartan and Hydrochlorothiazide.

Table No : 8

Inj. Sample	Spike level	Average area of 3 injections	%RSD	Amount	Amount recovered	% recovered	Mean recovery	Acceptance Criteria
Telmisartan	50 %	2380181	0.45	40µg	39.94µg	99.85%	99.85 %	98-102%
	100 %	7990697	0.35	80µg	79.96µg	99.95%		
	150 %	10110923	0.33	120µg	119.73 µg	99.77%		
Hydrochlorothiazide	50 %	924988	0.57	12.5µg	12.27µg	98.16%	98.62 %	98-102%
	100 %	3017884	0.27	25µg	24.91µg	99.64%		
	150 %	3992816	0.45	32.5µg	31.87µg	98.06%		

4.6.3. PRECISION

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation and relative standard deviation.

Determination:

The precision of the analytical method was determined by assaying sufficient number of samples and relative standard deviation was calculated.

The precision of the instrument was determined by assaying the samples consecutively, number of time and relative standard deviation was calculated.

Procedure:

A) System Precision:

Preparation of Standard Solution :

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent (ACN:MET)was added to two flasks and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions were filtered through 0.45µm membrane filter (Stock solutions).

From above solutions 5ml Telmisartan stock solution and 5ml of Hydrochlorothiazide were pipetted out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

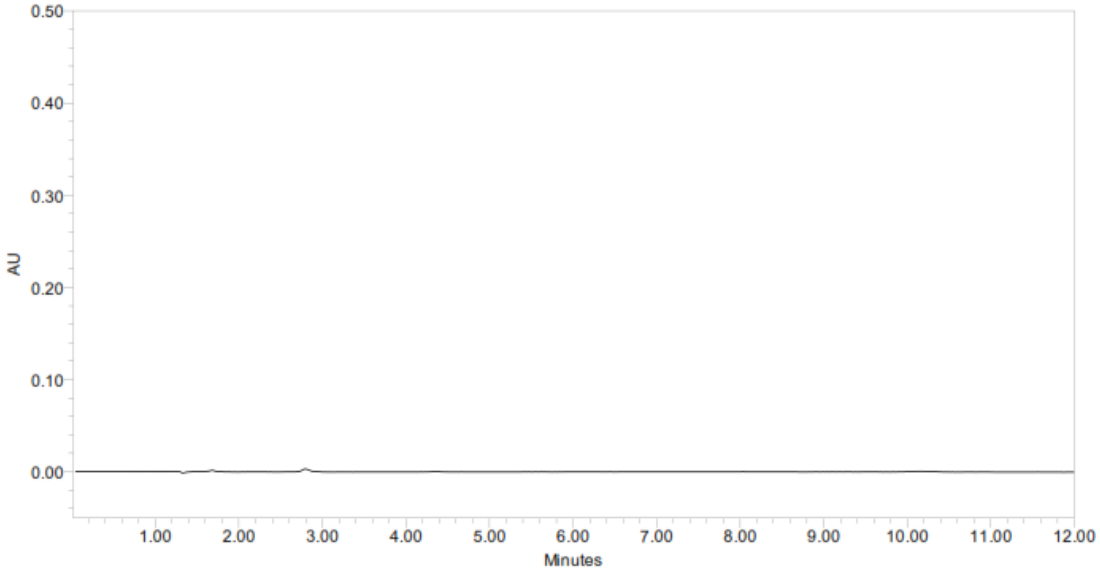
Inject 20µl of the blank solution and the standard solution of for five times and calculate the %RSD for the area of six replicate injections.The chromatograms are as shown in Fig No: 4.23-4.29 and the results are tabulated shown in Table No:9.

Blank solution: Mixture of trifluoro acetic acid (pH3) and acetonitrile in ratio of 70:30 was filtered and degassed.

System Precision:

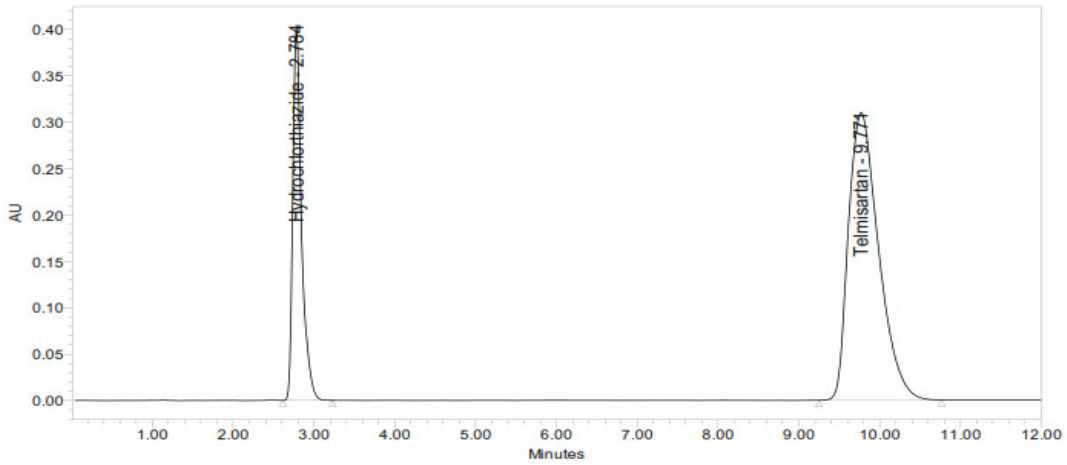
Chromatogram showing Precision-blank solution.

Fig No : 4.23



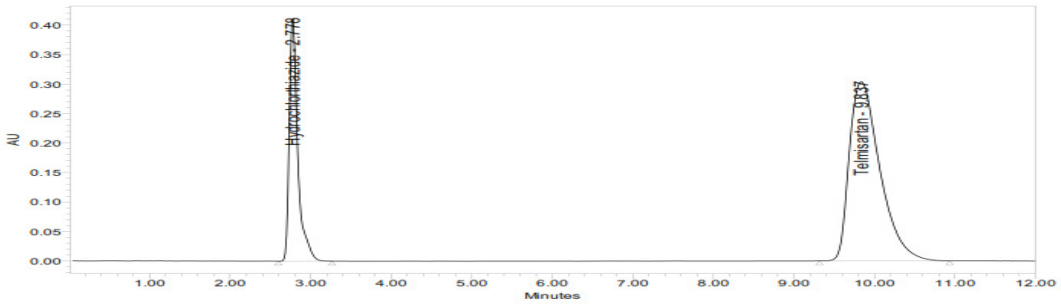
Chromatograms showing System Precision of Standard for Telmisartan and Hydrochlorothiazide

Fig No : 4.24



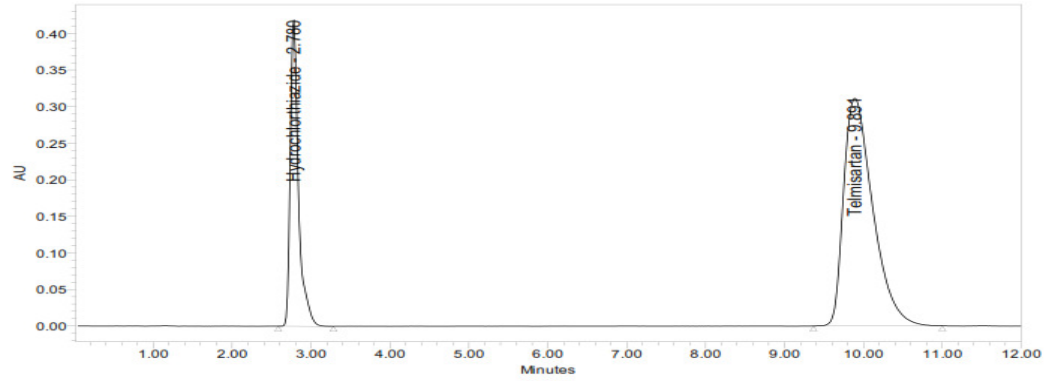
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.784	3022485	27.36	402996	1.5	3666
2	Telmisartan	9.771	8026557	72.64	310471	1.4	3138

Fig No : 4.25



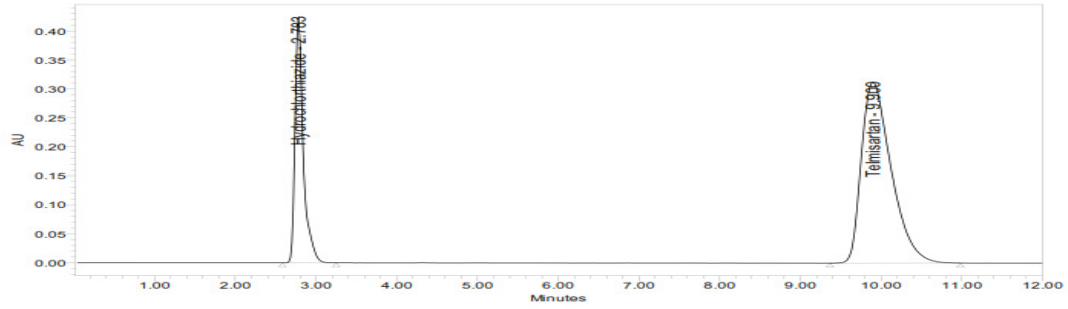
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.778	3028837	27.43	412784	1.7	3953
2	Telmisartan	9.837	8013447	72.57	304186	1.6	3312

Fig No : 4.26



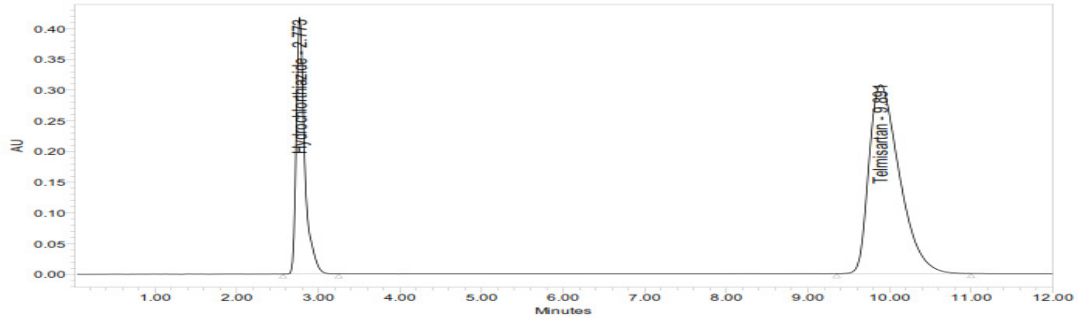
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.780	3018824	27.38	418189	1.6	4108
2	Telmisartan	9.891	8007693	72.62	311578	1.6	3418

Fig No :4.27



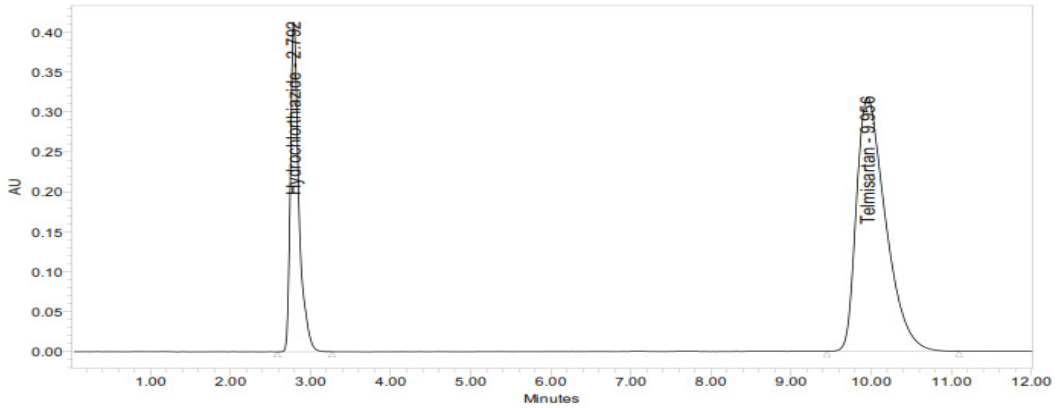
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.783	3022719	27.40	422399	1.6	4219
2	Telmisartan	9.900	8008710	72.60	312851	1.5	3368

Fig No :4.28



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.773	3004344	27.40	418971	1.6	3930
2	Telmisartan	9.891	7960524	72.60	307988	1.5	3312

Fig No : 4.29



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.792	3023273	27.38	417127	1.6	3878
2	Telmisartan	9.956	8019768	72.62	318441	1.5	3494

B) Method Precision:

Preparation of Sample Solution:

Accurately pipette out 5ml of the sample (equivalent to 0.8µg/ml of Telmisartan &0.25µg/ml of Hydrochlorothiazide) into a 50 ml volumetric flask and made up to the mark with mobile phase. Mix well and filter through 0.45µm filter.

Inject 50µl of the blank solution and six replicate injections of sample solution of 100 µg/ml for six times and calculate the %RSD for the area of six replicate injections.

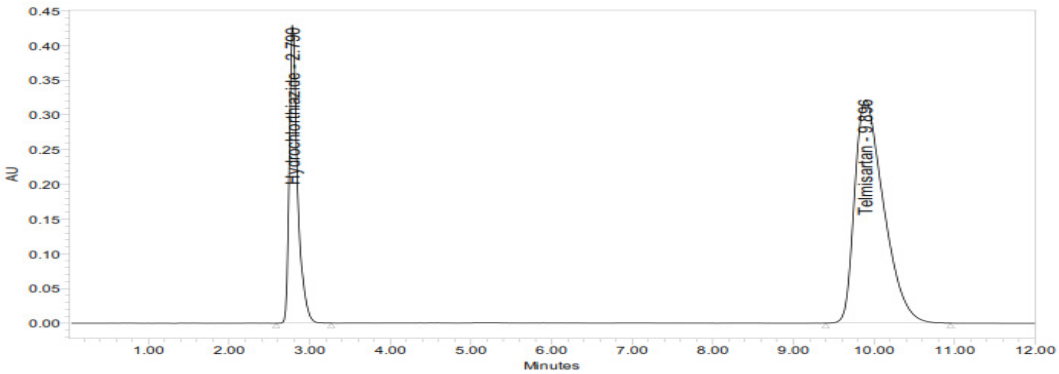
%RSD Formula: $(\sigma/\mu)*100$

Acceptance criteria: % Relative standard deviation (%RSD) for the areas of Telmisartan & Hydrochlorothiazide from the standard chromatograms should not be more than 2.0

The method precision was determined by preparing the sample from the tablet formulation for five times and six successive injections of 20µl of working sample solution were injected and the chromatograms were recorded and shown in Fig No:4.30 to 4.35 and method precision data are shown in table. 9

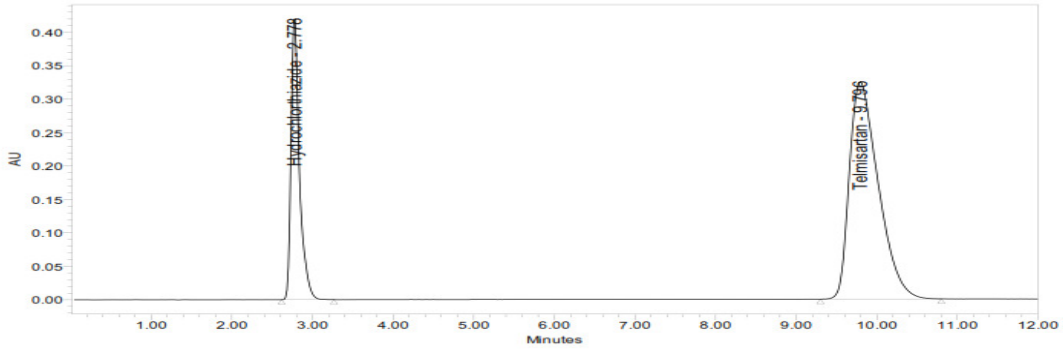
Chromatogram showing Method Precision of Telmisartan and Hydrochlorothiazide

Fig No : 4.30



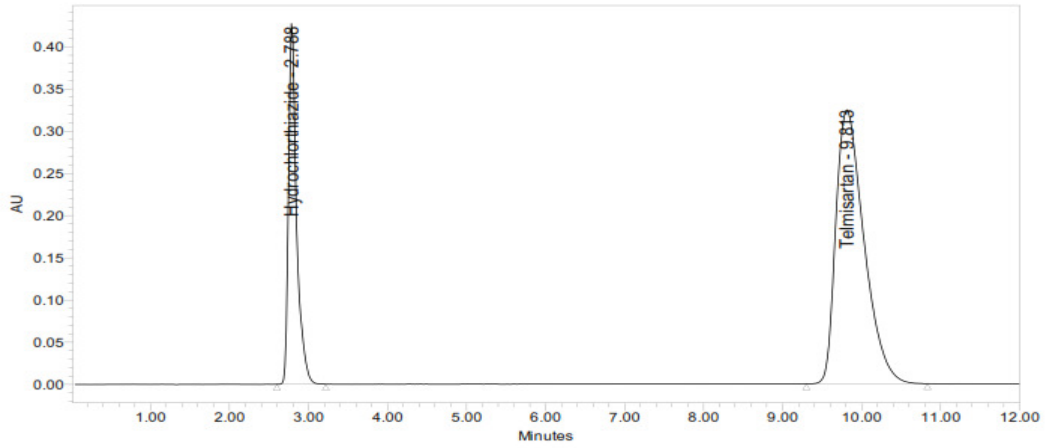
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.790	3033358	27.43	430068	1.5	3996
2	Telmisartan	9.896	8026804	72.57	321398	1.5	3453

FigNO:4.31



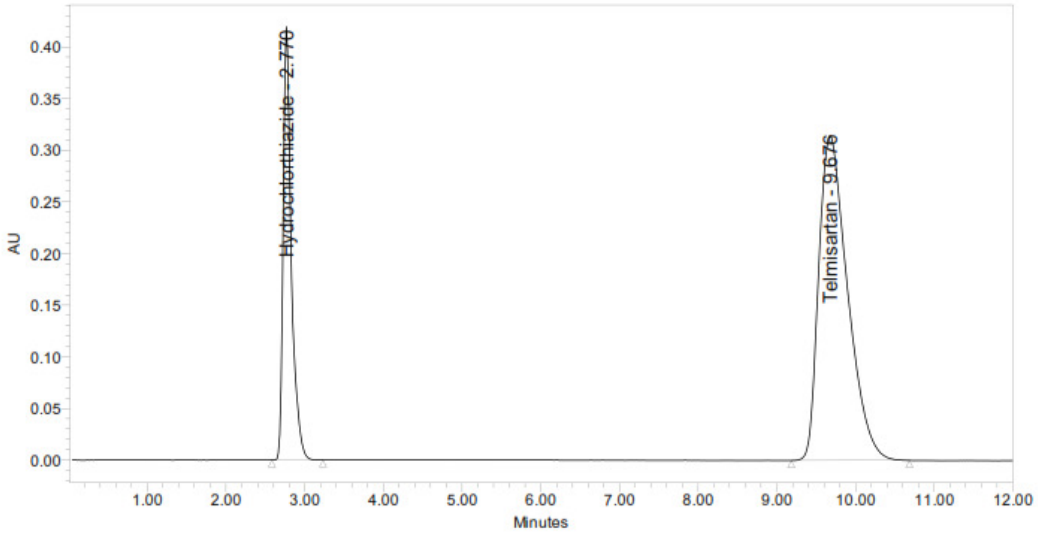
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.778	3048427	27.41	421187	1.5	3888
2	Telmisartan	9.796	8073243	72.59	325061	1.5	3349

FigNO:4.32



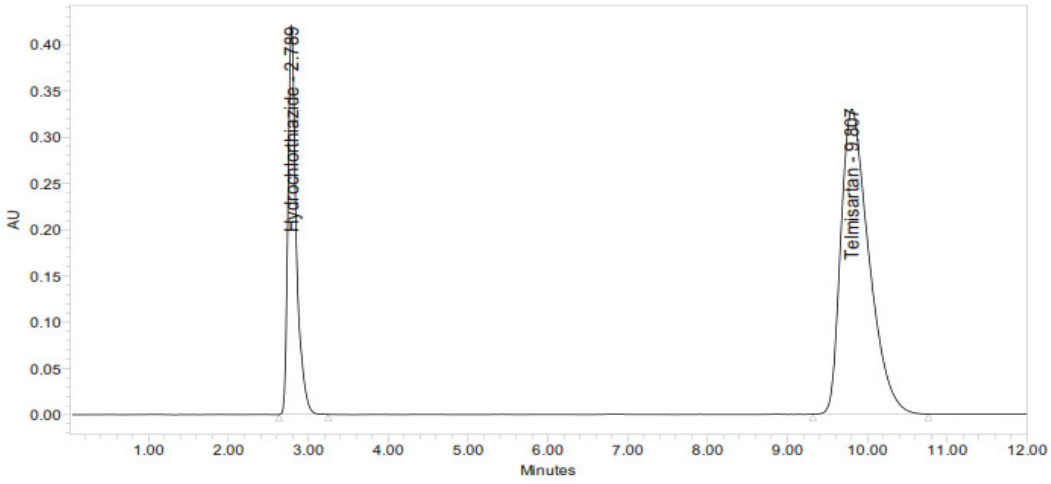
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.788	3027459	27.40	426268	1.6	4038
2	Telmisartan	9.813	8022156	72.60	324417	1.5	3554

FigNO:4.33



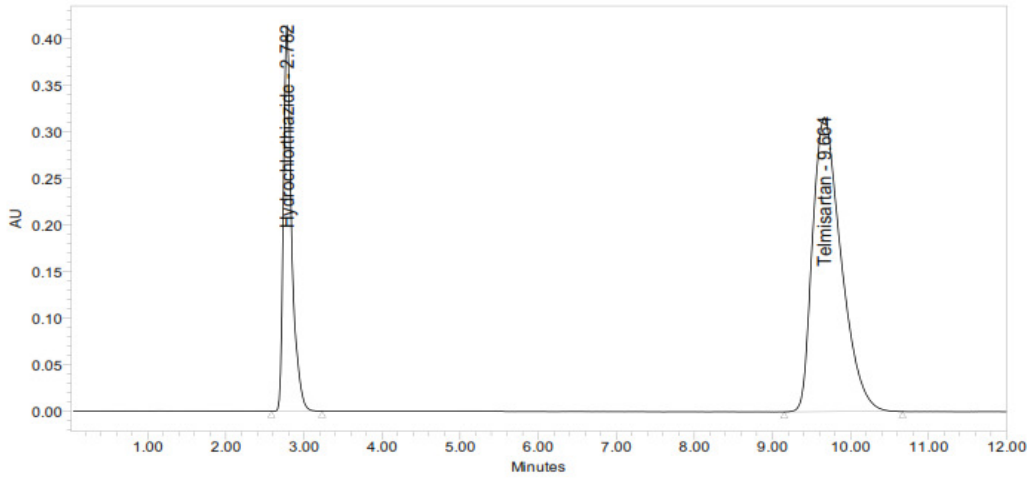
	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.770	3015367	27.42	417934	1.5	3906
2	Telmisartan	9.676	7982864	72.58	314538	1.5	3129

FigNO:4.34



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.789	3032022	27.45	421724	1.5	3877
2	Telmisartan	9.807	8012982	72.55	329872	1.5	3527

FigNO:4.35



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.782	3036347	27.46	412473	1.5	3909
2	Telmisartan	9.664	8020473	72.54	316151	1.5	3147

Table showing Results of Precision for Telmisartan and Hydrochlorothiazide

Table No : 9

Parameter	**Method Precision**		**System Precision**	
Average Area	**Telmisartan**	**Hydrochlorothiazide**	**Telmisartan**	**Hydrochlorothiazide**
	8025108	3032192	8003386	3019442
SD	32150.46	12121.06	25110.58	9173.278
%RSD	0.4	0.3	0.31	0.3

4.6.4. SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Procedure:

Preparation of placebo :

Placebo was prepared by mixing all the excipients without active ingredients.

Accurately 195 mg of placebo was weighed and transferred into 100ml volumetric flask, about 70ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 5ml of solution was pipetted out and transferred into 50ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of placebo solution into the HPLC system and chromatograph.
Compare the chromatograms visually and check for any interference.

Preparation of Standard solution :

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent (ACN:MET)was added to two flasks and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions were filtered through 0.45µm membrane filter (Stock solutions).

From above solutions 5 ml Telmisartan stock solution and 5ml of Hydrochlorothiazide were pipetted out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph.
Compare the chromatograms visually and check for any interference.

Preparation of Standard + placebo:

Accurately weighed quantity of placebo transferred into two 100 ml flasks. To this accurately weighed quantity of 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide weighed separately and transferred into flasks. To this about 70 ml of diluents was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

From this 5 ml of solution was pipette out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference.

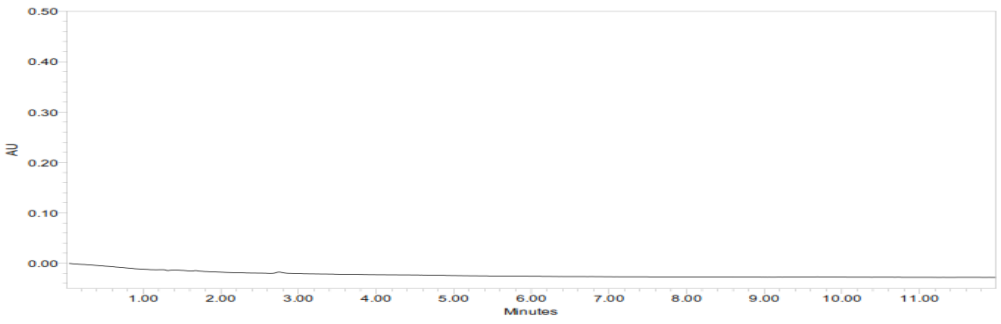
%RSD Formula: (σ /µ)*100

Acceptance criteria:

There should not be any peak in the blank and Placebo solution run at the retention time corresponding to Telmisartan and Hydrochlorothiazide as in standard run.

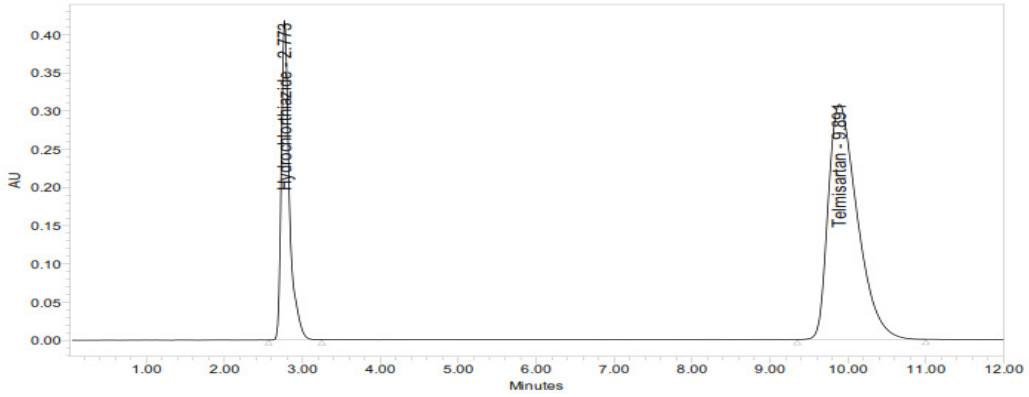
Chromatogram showing Specificity- Blank solution

Fig No : 4.36



Chromatogram showing Specificity-Standard solution of Telmisartan and Hydrochlorothiazide.

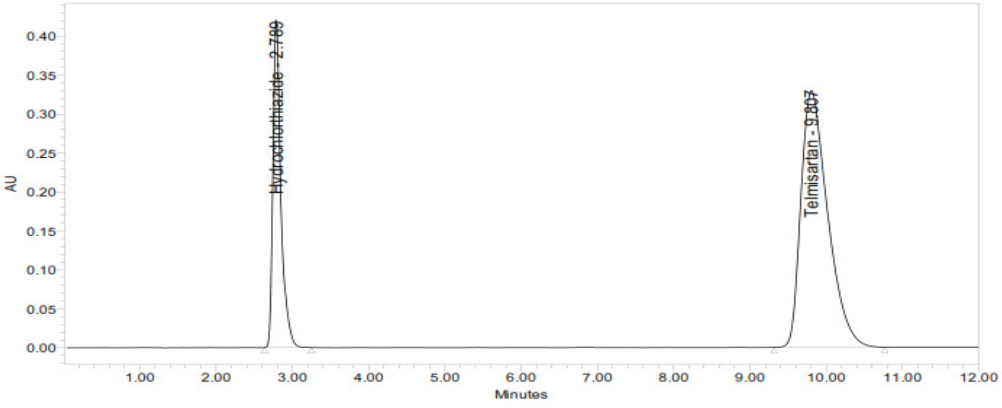
Fig No : 4.37



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.773	3004344	27.40	418971	1.6	3930
2	Telmisartan	9.891	7960524	72.60	307988	1.5	3312

Chromatogram showing placebo+standard solution

Fig No : 4.38



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.789	3032022	27.45	421724	1.5	3877
2	Telmisartan	9.807	8012982	72.55	329872	1.5	3527

4.6.5. LINEARITY&RANGE

LINEARITY: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

Range: Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrated that the analytical procedure has a sutable level of precision, accuracy, and linearity.

Procedure:

Preparation of Standard Stock Solution:

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent was added and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter (Stock solution).

Preparation of sample solutions:

From the above stock solution pipette out 0.5, 1, 2.5, 5.0,6.0 and7.5ml respectively into individual 50ml of volumetric flasks and diluted up to the mark with diluent to prepare 10,20,50,100,120,150% sample solutions respectively. Mix well and filter through 0.45µm filter.

Inject 20µl of blank solution and each linearity level standard solutions into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are tabulated shown in Table No: 11

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

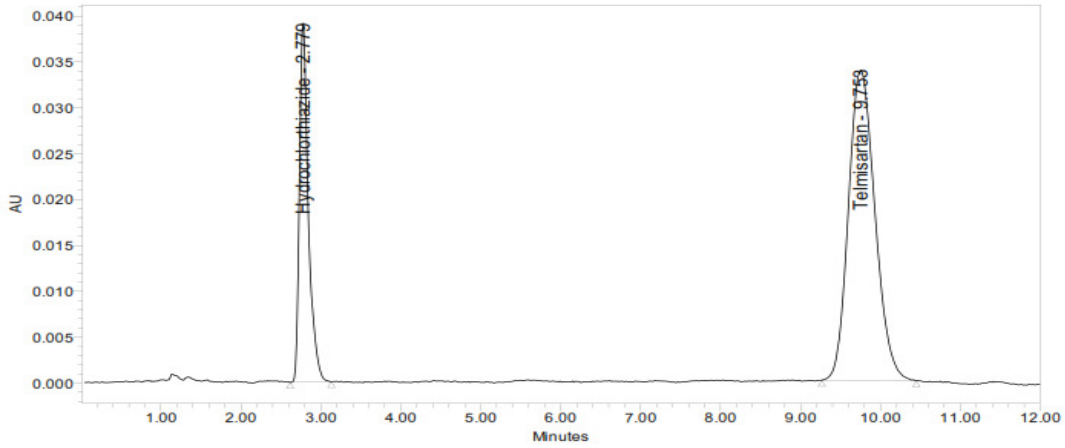
Acceptance criteria for Range:

- Correlation coefficient should not be less than 0.99%

The linearity data and analytical performance parameters of are shown in table and calibration curve of is shown Fig No: 4.39 to 4.44.

Chromatogram showing Linearity level-1 (10%) Telmisartan and Hydrochlorothiazide

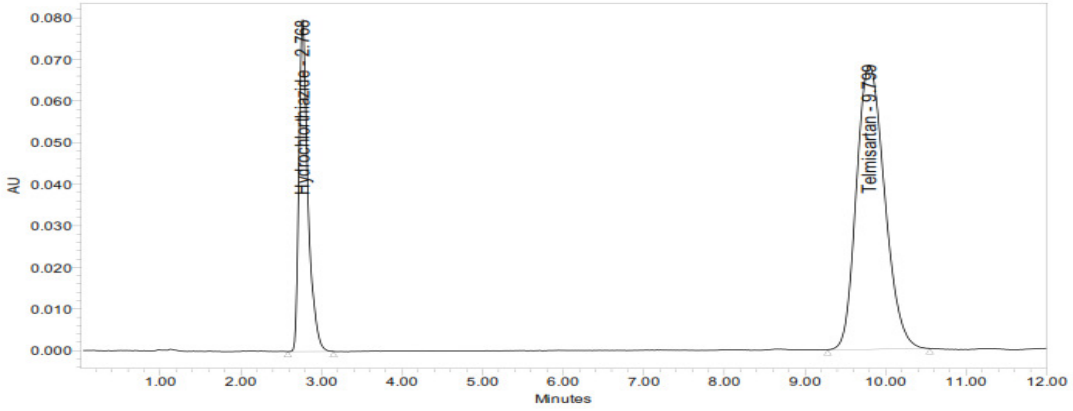
Fig No : 4.39



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.779	301937	27.93	39153
2	Telmisartan	9.753	779093	72.07	33839

Chromatogram showing Linearity level-2 (20%) of Telmisartan and Hydrochlorothiazide

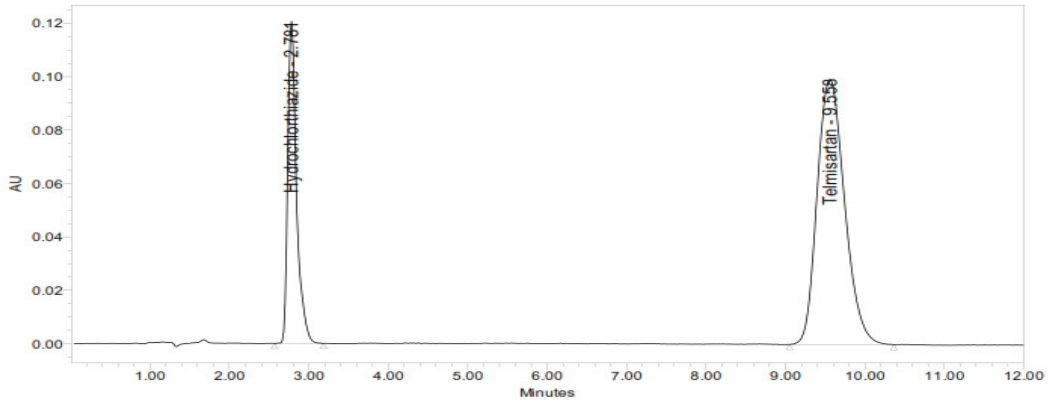
Fig No: 4.40



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.768	620422	27.21	79563
2	Telmisartan	9.799	1659459	72.79	68314

Chromatogram showing Linearity level-3 (50%) of Telmisartan and Hydrochlorothiazide.

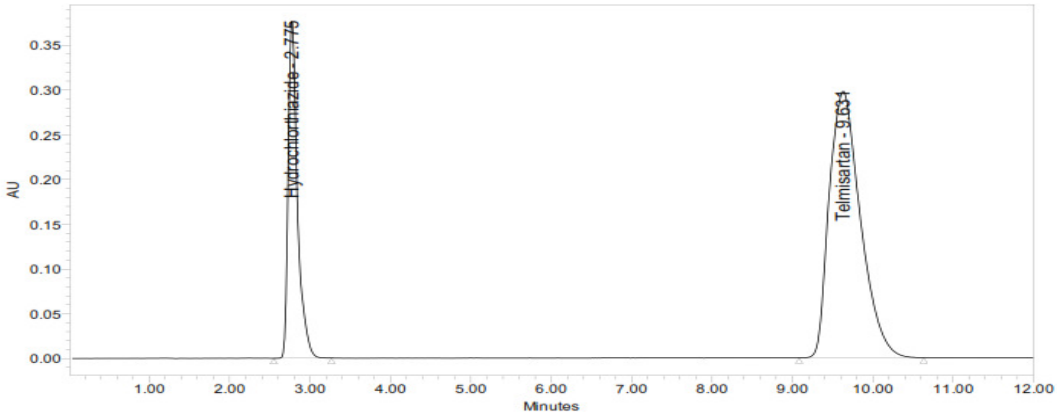
Fig No : 4.41



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.781	1512794	28.02	120402
2	Telmisartan	9.558	4005821	71.98	99267

Chromatogram showing Linearity level-4 (100%) of Telmisartan and Hydrochlorothiazide.

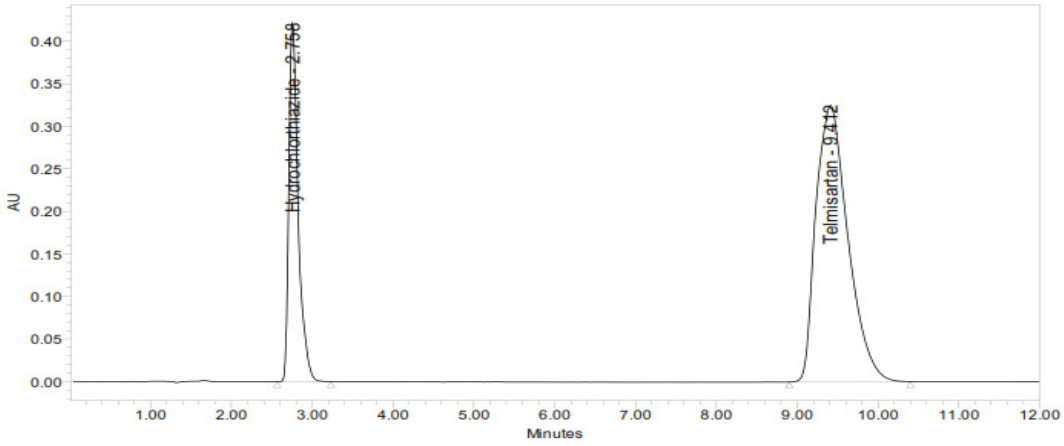
Fig No : 4.42



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.775	3025588	27.41	381000
2	Telmisartan	9.631	8011642	72.59	297956

Chromatogram showing Linearity level-5 (120%) of Telmisartan and Hydrochlorothiazide.

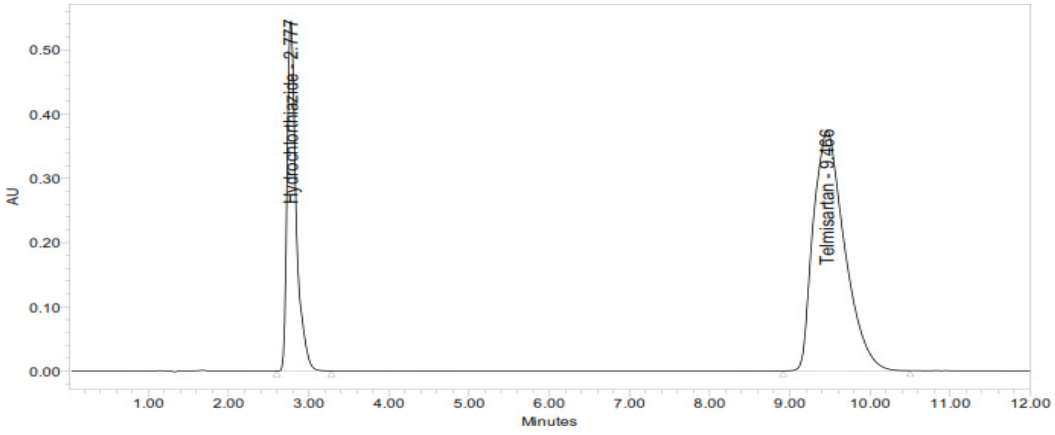
Fig No : 4.43



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.758	3630705	27.52	426050
2	Telmisartan	9.412	9613970	72.48	325273

Chromatogram showing Linearity level-6 (150%) of Telmisartan and Hydrochlorothiazide.

FigNO:4.44



	Peak Name	RT	Area	% Area	Height
1	Hydrochlorthiazide	2.777	4438382	29.72	547036
2	Telmisartan	9.466	11817460	70.28	374310

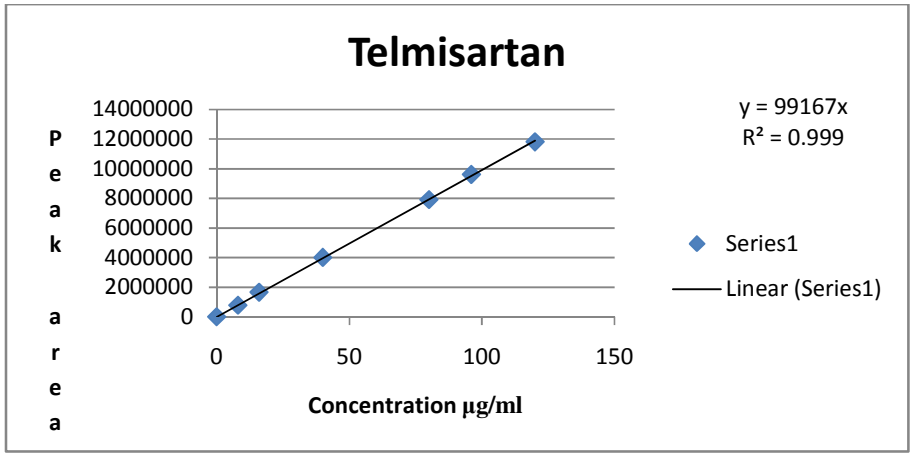
Linearity Results (for Telmisartan):

Table No : 10

S. No	Linearity Level	Concentration	Area
1	I	8µg/ml	779093
2	II	16µg/ml	1659459
3	III	40µg/ml	4005821
4	IV	80µg/ml	8011642
5	V	96µg/ml	9613970
6	VI	120µg/ml	11817460
Correlation Coefficient			0.9998

Figure showing Linearity graph of Telmisartan.

FigNO:4.45



The correlation coefficient value was found to be 0.999

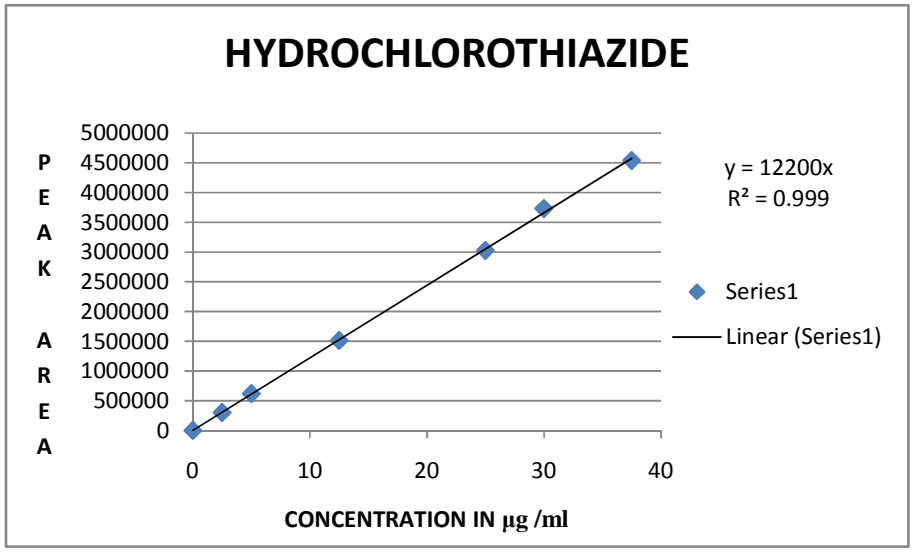
Linearity Results for Hydrochlorothiazide:

Table No : 11

S. No	Linearity Level	Concentration	Area
1	I	2.5µg/ml	301937
2	II	5.0µg/ml	620422
3	III	12.5µg/ml	1512794
4	IV	25µg/ml	3025588
5	V	30µg/ml	3630705
6	VI	37.5µg/ml	4438382
Correlation Coefficient			0.9996

Figure showing linearity graph of Hydrochlorothiazide.

Fig No : 4.46



The correlation coefficient value was found to be 0.9996

4.6.6 ROBUSTNESS

It is a measure of ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but were still within the specified parameters of the assay for example change in physical parameters like flow rate, column temperature and mobile phase ratio.

Preparation of Standard Solution :

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent (Acetonitrile:Methanol)was added to two flasks and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions were filtered through 0.45µm membrane filter (Stock solutions).

From above solutions 5 ml Telmisartan stock solution and 5ml of Hydrochlorothiazide were pipetted out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of the blank solution and the standard solution for five times and analysed using varied flow rates (1.2ml, 0.8 ml) along with method flow rate and calculate the %RSD for the area of five replicate injections.

a) The flow rate was varied at ±0.2 ml/min.

%RSD of Telmisartan and Hydrochlorothiazide assay under these conditions is calculated and the results are shown in below.

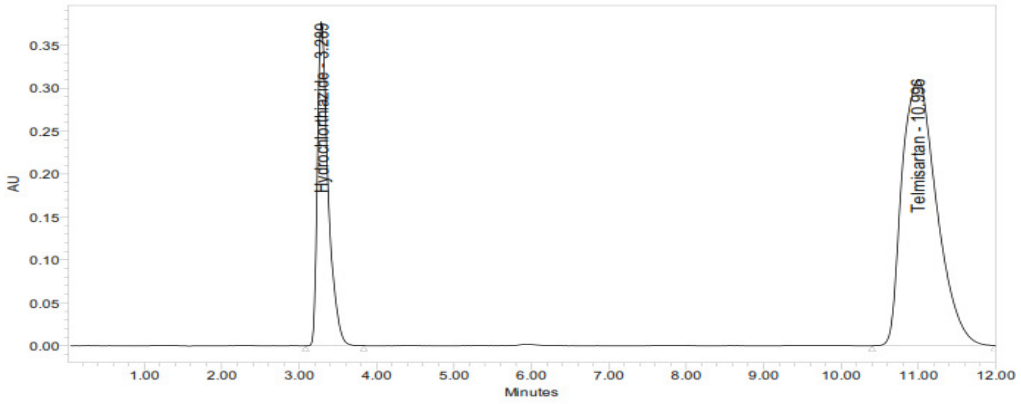
PROCEDURE

a) **The flow rate was varied at ±0.2 ml/min.**

Standard solution was prepared and analysed using the varied flow rates along with method flow rate and the chromatograms were recorded. The results are shown in table 12.

Chromatogram showing Robustness-Flow variation (0.8ml/min) of Telmisartan and Hydrochlorothiazide.

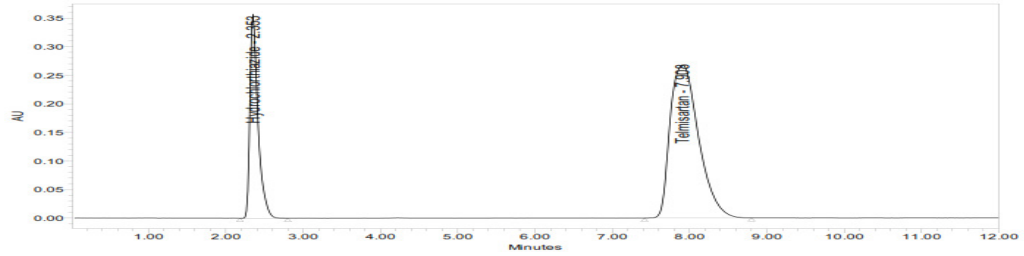
Fig No : 4.47



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	3.289	3629910	27.42	378975	1.6	3007
2	Telmisartan	10.996	9609688	72.58	310199	1.3	2909

Chromatogram showing Robustness-Flow variation (1.2ml/min) of Telmisartan and Hydrochlorothiazide.

Fig No :4.48



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.353	2585038	27.42	356128	1.6	2605
2	Telmisartan	7.908	6842795	72.58	268143	1.4	2393

B) COLUMN TEMPERATURE:

Preparation of Standard Solution :

Accurately 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide were weighed separately and transferred into two 100ml volumetric flasks, about 70ml of diluent (ACN:MET)was added to two flasks and sonicated for 5 minutes to dissolve it. The volume was made up with mobile phase. The solutions were filtered through 0.45µm membrane filter (Stock solutions).

From above solutions 5 ml Telmisartan stock solution and 5ml of Hydrochlorothiazide were pipetted out and transferred into 50 ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 20µl of the blank solution and the standard solution for five times and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method and calculate the %RSD for the area of five replicate injections. The chromatograms are as shown in Fig No: 4.48 to 4.49 and the results are tabulated shown in Table No: 13.

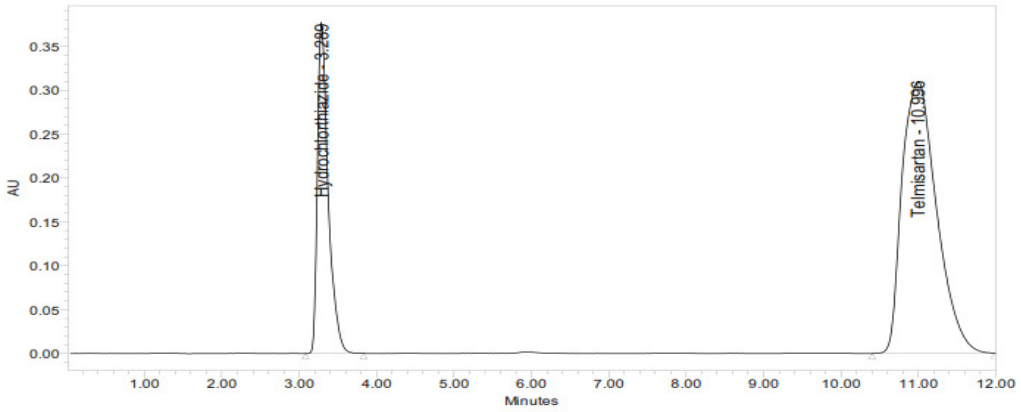
%RSD Formula: (σ /µ)*100

Acceptance criteria:

Relative standard deviation (RSD) of areas of Telmisartan and Hydrochlorothiazide from five standard chromatograms in all the flow rate variation and mobile phase composition should not be more than 2.0 %.

ROBUSTNESS COLUMN TEMPERATURE MINUS (-5˚C)

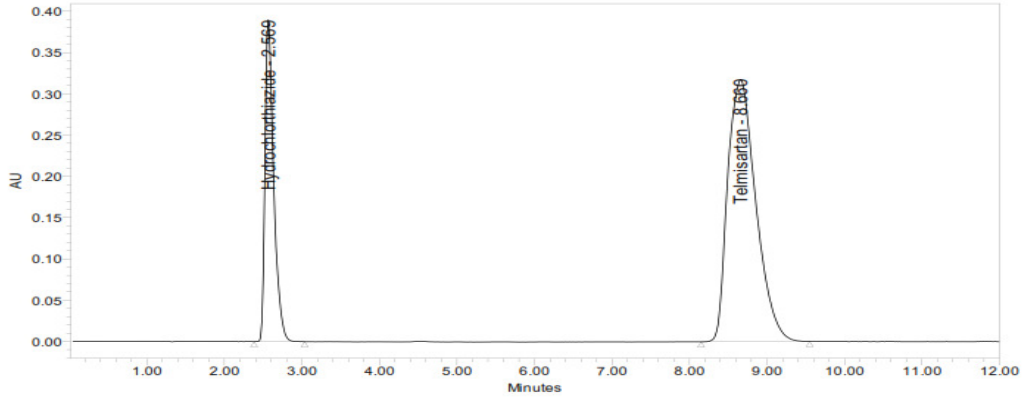
Fig No :4.49



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	3.289	3629910	27.42	378975	1.6	3007
2	Telmisartan	10.996	9609688	72.58	310199	1.3	2909

ROBUSTNESS COLUMN TEMPERATURE PLUS (+5˚C)

Fig No: 4.50



	Peak Name	RT	Area	% Area	Height	USP Tailing	USP Plate Count
1	Hydrochlorthiazide	2.569	3029841	27.44	389648	1.6	2649
2	Telmisartan	8.660	8011693	72.56	317564	1.3	2673

Table showing Robustness results for change in flow rate and mobile phase of Telmisartan and Hydrochlorothiazide.

Table No : 13

Flow rate	**Inj. Sample**	**Area**	**Plate count**	**Tailing**	**RT**
0.8ml/min	**Telmisartan**	9609688	2909	1.3	10.99
	Hydrochlorothiazide	3629910	3007	1.6	3.289
1.2ml/min	**Telmisartan**	6842795	2393	1.4	7.908
	Hydrochlorothiazide	2585038	2605	1.6	2.353
Column temperature variation					
+5˚C	**Telmisartan**	8011693	2673	1.3	8.660
	Hydrochlorothiazide	3029841	2649	1.6	2.569
-5˚C	**Telmisartan**	9609688	2909	1.3	10.996
	Hydrochlorothiazide	3629910	3007	1.6	3.289

4.7. Forced degradation studies:

Forced degradation studies were carried out on the sample preparation of Telmisartan and Hydrochlorothiazide and the degradation was evaluated by calculating the % degradation of Telmisartan and Hydrochlorothiazide in comparison with unstressed sample preparation.

The following are the stress conditions which were followed for forced degradation studies:

1. Acid Degradation
2. Alkali Degradation
3. Thermal Degradation
4. Photolytic

4.7.1 Acid Stress degradation:

Test preparation was subjected to acid stress degradation by treating the sample with hydrochloric acid . The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample.

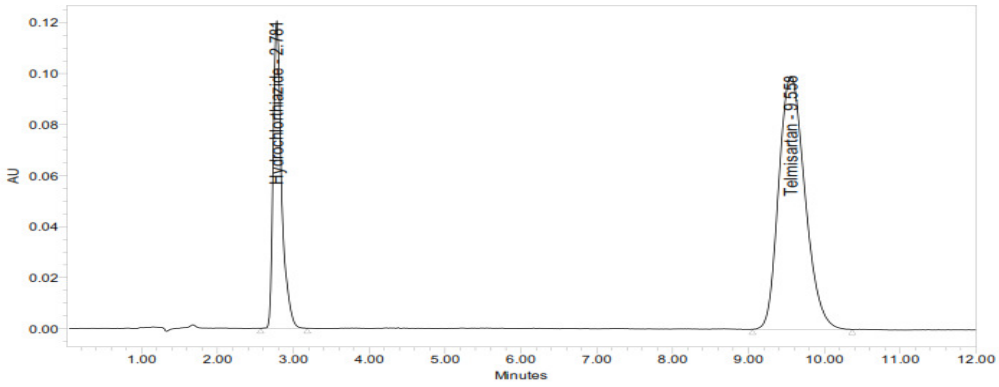
Test preparation:

Treated with 2N HCL:

Weighed and transferred sample equivallent to 80 mg Telmisartan and 25 mg of Hydrochlorothiazide into 100 ml volumetric flask, added 70ml of diluent a nd sonicated for 5 minutes.Then take 5 ml and transferred into 50 ml volumetric flask , added 30 ml of diluent with intermittent shaking, 5ml of 2N Hydrochloric acid is added and heated for 2hrs on a water bath at 80°C. Cool and neutralized with 5ml of 2N sodium hydroxide.

Chromatogram showing degradation of telmisartan and hydrochlorothiazide by acid

FigNO:4.51



4.7.2 Alkali stress degradation:

Test preparation was subjected to alkali stress degradation by treating the sample with sodium hydroxide. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample.

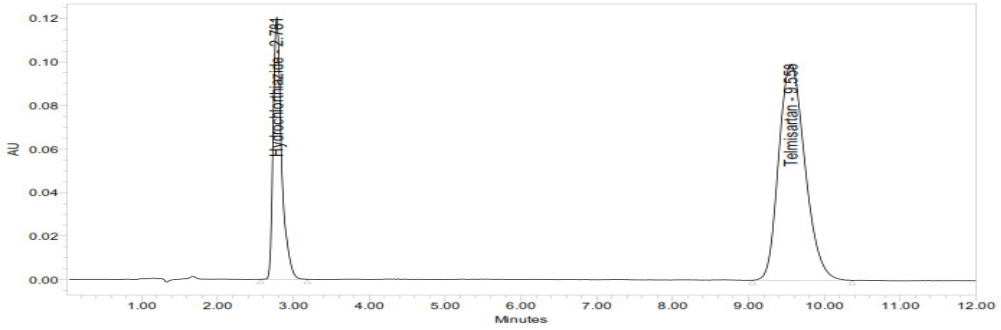
Test preparation:

Treated with 2N NaOH:

Weighed and transferred sample equivalent to 80 mg of telmisartan and 25 mg of Hydrochlorothiazide into 100 ml volumetric flask. Then transferred 5 ml of this solution into 50 ml of volumetric flask and added 30 ml of diluent and sonicated for 5 minutes with intermittent shaking, 5ml of 2N NaOH is added and heated for 2hrs on a water bath at 80°C. Cool and neutralized with 5ml of 2N HCL.

Chromatogram showing degradation of telmisartan and hydrochlorothiazide by base

Fig NO:4.52



4.7.3 Thermal stress degradation:

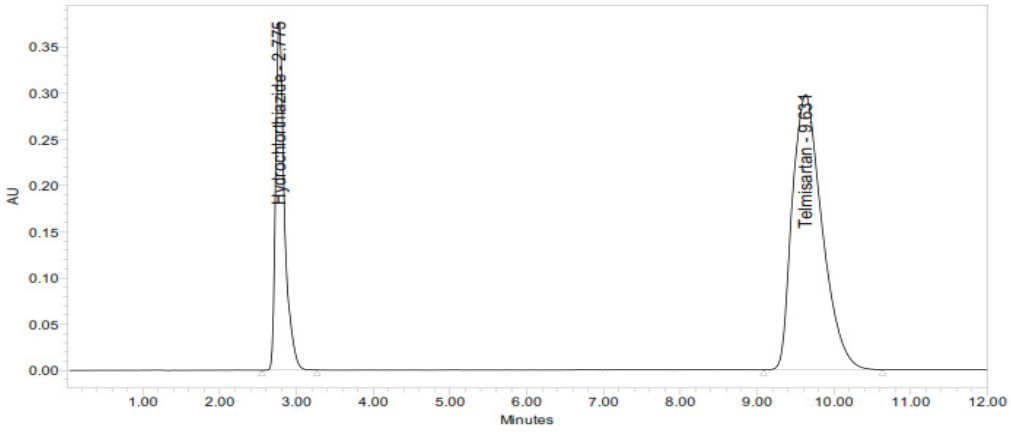
Test preparation was subjected to thermal treatment (80°C) for sufficient time. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample.

Test preparation:

Weighed and transferred sample equivalent to 80 mg Telmisartan and 25 mg Hydrochlorothiazide into 100 ml volumetric flask, then added diluents 70 ml and make upto the mark. Then 5 ml of this transferred into 50 ml volumetric flask.(which was kept in hot air oven previously at 80°C for 3 hours) added diluents and sonicate for 5 minutes and made upto the mark.

Chromatogram showing degradation of telmisartan and hydrochlorothiazide by thermal stress

Fig NO:4.53



4.7.4 Photolysis:

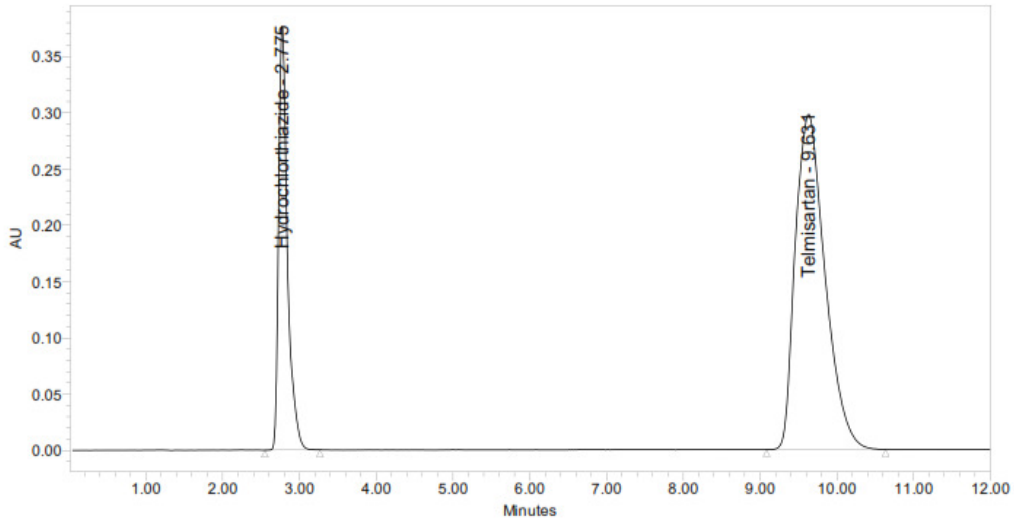
Test preparation was subjected to UV light 365nm for 8hrs. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample.

Test preparation:

Weighed and transferred sample equivalent to 80 mg of Telmisartan and 25 mg of Hydrochlorothiazide into 100 ml volumetric flask.Then make upto the mark and then take 5 ml of this solution and transferred into 50 ml volumetric flask. Then about 30 ml of diluents was added and sonicate for 5 minutes andmade upto the mark with diluent and mixed well and kept aside under the UV light at 271nm for 8hrs. Inject 10µl of sample solution into HPLC system to obtain chromatogram.

Chromatogram showing degradation of telmisartan and hydrochlorothiazide by light

Fig NO:4.54



% DEG formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{\text{average wt}}{L.C} \times \text{potency of drug}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

Table showing %Degradation of Telmisartan and Hydrochlorothiazide

Table.NO.13

S.NO	Degradation parameter	Telmisartan			Hydrochlorothiazide		
		Peak Area	%DEG	%Assay	Peak Area	%DEG	%Assay
1.	Acid	7812474	2.3	96.7	2912560	3.9	95.1
2.	Base	5083632	29.5	69.5	2474534	14.6	84.3
3.	Thermal	7865379	1.8	97.7	2919833	1.2	97.8
4.	Photolytic	79657456	2.2	96.8	2819425	2.3	96.7

RESULTS AND DISCUSSION

In the present work a new method development and validation was carried out for the estimation of Telmisartan and Hydrochlorothiazide by RP-HPLC technique. The wavelength selection was made at 271 nm since all the two compounds maximum absorbance in UV spectrum as reported in the literature is in 271 nm.

5.1.Method development: : For the method development several trials were carried out and reported. These leads to the optimized chromatographic conditions for the estimation of Telmisartan and Hydrochlorothiazide in pharmaceutical dosage form. By Inertsil ODS 3V (4.6mm×150,5µm) column eluted with mobile phase acetonitrile : trifluoro acetic acid buffer (30:70) at a flow rate of 1 ml/ min and a detection wavelength of 271 nm with injection volume of 20 µl at ambient(25°C) temperature afforded the best results.

FIXED CHROMATOGRAPHIC CONDITION

Table No: 14

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	isocratic elution
Mobile phase	Solvent-A: Trifluoro acetic acid pH-3 Solvent-B: ACN(70:30)
Column	Inertsil ODS 3V (4.6 x 150mm, 5 µm)
Flow rate	1 mL/ min
Detection Wavelength	271 nm
Injection volume	20 µl
Column oven temperature	Ambient(30°C)
Run time	12 min

Benefits of my method compared to previous methods:

The retention times of Telmisartan and Hydrochlorothiazide were 2.7 and 9.8 mins. And no internal standard used for this estimation. The calibration curve shows excellent linearity over the concentration range for Telmisartan and hydrochlorothiazide were 8-120µg/ml and 2.5-37.5µg/ml.The mobile phase ratio of buffer and Acetonitrile was 70:30.

So this method is said to be economical and sensitive.

Table showing comparison of previous method to proposed method:Table NO:15

Method	Column	Mobile Phase	Mobile phase ratio	pH	Linearity range	Retension Time
Reported method 1	ACE C_{18} (250cm ×4.6)	Sodium perchlorate monohydrate: Acetonitrile	55:45	3.0 Adjusted with trifluoro acetic acid	Telmisartan-140-260µg/ml and Hydrochlorothiazide17. 5-32.5µg/ml	Telmisartan-11.26 Hydrochlorothiazide-3.17
Reported method 2	Hypersil gold(25 cm×4.6 mm)	Potassium dihydrogen phosphate: Acetonitrile	65:35	5.5	Telmisartan-24.12-56.27µg/ml Hydrochlorothiazide-6-22.4µg/ml	Telmisartan-11.72 Hydrochlorothiazide-3.71
Proposed method	Inertsil ODS (4.6x150 mm ,5µm	Trifluoro acetic acid : Acetonitrile	70:30	3.0	Telmisartan-8-120 µg/ml and Hydrochlorothiazide – 2.5-37.5µg/ml	Telmisartan - 9.8 Hydrochlorothiazide-2.73

5.2. METHOD VALIDATION:

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination, which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

Table showing Summary of results of method validation for Telmisartan and Hydrochlorothiazide.Table no 16

S. No	Parameter	Requirement	results		Acceptance criteria
			Telmisartan	**Hydrochlorothiazide**	
	System suitability	RT	9.771	2.784	
1.		Tailing factor	1.4	1.5	NMT 2
		Plate count	3340.33	3959	NLT 2000
		%RSD	0.35	0.4	
2.	Assy	Assay value	99.56%	99.1	98-102%
3.	Accuracy	% Recovery	98.965	98.84%	98-102%
4.	System Precision	%RSD	0.31	0.3	NMT 2%
	Method Precision	%RSD	0.4	0.39	NMT2%
5.	Specificity	No interference	Pass	Pass	No interference
6.	Linearity	Correlation coefficient	0.999	0.999	NLT 0.999
7.	Range	Concentration	8-120µg/ml	2.5-37.7µg/ml	Nil
8.	Robustness	%RSD	0.35	0.37	NMT 2%
9.	Forced degradation Acid Base Thermal Photolytic	%degradation	Within the limit	Within the limit	NMT 30%

a.System suitability:

From the system suitability studies it was observed that % RSD of retention times were found to be 0.35%(Telmisartan) and 0.4%(Hydrochlorothiazide). USP tailing factor was found to be 1.4(Telmisartan) and 1.5(Hydrochorothiazide). Average theoretical plates are found to be 3340.33(telmisartan) and 3959(Hydrochlorothiazide). All the parameters were within the limit.

b.Linearity:

From the Linearity data it was observed that the method was showing linearity in the concentration range of 8-120 µg / ml for Telmisartan and 2.5-37.5µg/ml for Hydrchlorothiazide. Correlation coefficient was found to be 0.9998 for Telmisartan and 0.9996 forHydrochlorothiazide.

c.Precision: The RSD of peak area for standard chromatograms of system precision were found to be 0.31 % for Telmisartan and 0.3% for Hydrochlorothiazide , and in method precision it was found to be 0.4% for Telmisartan and 0.39% for Hydroclorothiazide. It passes method and system precision.

d.Accuracy: The percentage recovery of telmisartan and hydrochlorothiazide were 98.964 and 98.84 %.The percentage recovery of the two drugs were within the limit.

e.SPECIFICITY:

The chromatograms of standard and sample are identical with nearly same retention time. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific.

f.Robustness:

As the % RSD of retention time and asymmetry were within limits for variation (± 5˚c) in column temperature. Hence the allowable variation in wavelength was ± 5˚c.

As the % RSD of retention time and asymmetry were within limits for variation in flow rate (± 0.2 ml). Hence the allowable flow rate should be within 0.8 ml to 1.2 ml.

g.Forced degradation studies:

The % degradation of Telmisartan and Hydrochlorothiazide by various conditions like acid,base,thermal,photolytic were performed .The results of these studies were

Acid stress degradation:

The % degradation of Telmisartan was 2.3%. The % degradation of Hydrochlorothiazide was 3.9.The % degradation of two drugs were within the limit.

Alkali stress degradation:

The % degradation of Telmisartan was 29.5%.The % degradation of hydrochlorothiazide was 14.6%. The % degradation of two drugs by alkali stress were within the limit.

Thermal stress degradation:

The % degradation of Telmisartan was 1.8%.The % degradation of Hydrochlorothiazide was 1.2%. The % degradation of two drugs by thermal stress were within the limit.

Photolytic degradation:

The % degradation of Telmisartan was 2.2%.The % degradation of Hydrochlorothiazide was 2.3%. The % degradation of two drugs by photolysis were within the limit.

CONCLUSION

- A new method is developed for Simultaneous Estimation of Telmisartan and Hydrochlorothiazide by RP-HPLC method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH Q2B guidelines and shown to be accurate, precise and specific and economic and stability indicating.

- This method represents an economic and stability indicating analytical procedure for the simultaneous quantization of Telmisartan and Hydrochlorothiazide. The method is amenable to the routine analysis of large numbers of samples with good precision and accuracy.

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